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ORIGINAL PAPER

Temporal dynamics of total and active prokaryotic communities in two Mediterranean orchard soils treated with solid anaerobic digestate or managed under no-tillage

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Abstract

A field experiment was carried out to investigate the impact of two improved tillage systems (conventional tillage combined with the incorporation of solid anaerobic digestate, no-tillage) on the prokaryotic community composition in two tree orchard (olive, citrus) soils with contrasting texture, carbonate content, and pH, located in Southern Italy. Soil samples were taken over a 5-month period to assess immediate (2 days) *vs* short-term (7 and 18 weeks) responses. Phylogenetic diversity and compositional shifts of both total and metabolically active soil prokaryotic communities were assessed by next-generation sequencing of 16S rRNA gene templates from soil-extracted DNA/RNA. In both digestate-treated soils, copiotrophic *α-Proteobacteria* and oligotrophic Acidobacteria, Gemmatimonadetes, and Verrucomicrobia showed an immediate (2 days) but short-lived (7 weeks) shift in their relative abundance similar in persistence but not in magnitude; whereas selective soil type-dependent responses were observed for Actinobacteria, Chloroflexi, Firmicutes, and Planctomycetes. The autochthonous soil microbiota demonstrated resilience to the addition of the anaerobic digestate, which was dominated by Firmicutes, Bacteroidetes, Deinococcus-Thermus, and Euryarchaeota (Methanomicrobia). Likewise, a temporary increase in the relative abundances of copiotrophic taxa (Firmicutes, Bacteroidetes, Thaumarchaeota) was observed under conventional tillage, especially in the sandy loam (citrus) soil. Conversely, no-tillage favored the establishment of oligotrophic Chloroflexi and Verrucomicrobia in both soils. The active and the total prokaryotic communities differed from each other only in physically disturbed soils. Soil management induced compositional shifts in the predominant microbial copiotrophic/oligotrophic community balance, whose persistence was linked to the tillage system, while magnitude depended on soil type.

Keywords 16S rDNA sequencing · *Citrus sinensis* (L.) Osbeck · *Olea europaea* L. · *Escherichia* spp. · r/K-strategists · Soil metagenomics

Introduction

According to estimates from the Global Assessment of Soil Degradation (GLASOD), a decline in soil quality accounts for an average of 9% reduction in soil productivity, while up to half of the world's agricultural land is degraded to some degree due to poor agricultural practices (Gibbs and Salmon 2015). This becomes particularly true under semi-arid Mediterranean conditions where agricultural intensification and conventional plow tillage often combined with no organic fertilization result in decreasing fertility, crop yield reduction, and, ultimately, arable land abandonment (Pagliai et al. 2004; Álvaro-Fuentes et al. 2007; Tóth et al. 2008; Zdruli et al. 2010).

In the Mediterranean area, perennial crops (olive, citrus, almond, grapevines, peach, apricot) represent ~ 16% of the agroecosystems with relevant economic importance. In most cases, fruit tree cultivation is traditionally characterized by mono-cropping with long tree spacing, mostly rainfed farming, and frequent tillage to avoid growth of weeds in the alley, thus leaving the soil bare for most of the year

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(Morugán-Coronado et al. 2020). These conditions make tree orchard soils highly vulnerable to loss of fertility, erosion, and degradation (Parras-Alcántara et al. 2016; Montanaro et al. 2017; Vignozzi et al. 2019).

The growing awareness of human-induced degradation processes as major threats to non-renewable soil resources has led to the development of conservative management practices of arable lands to improve soil structure and reduce erosion, raise the soil organic matter levels, restore the nutrient balance, sustain soil biodiversity, and mitigate greenhouse gas emissions (Holland 2004; Lal and Stewart 2013; Paustian et al. 2016; Martínez-Mena et al. 2020). The application of organic amendments, such as the newly appeared digestates, is also greatly beneficial to soil fertility, especially in European semi-arid Mediterranean regions (Diacono and Montemurro 2010). Originating as by-products of the biological process of the anaerobic digestion (AD) of mixed organic wastes for the production of a renewable fuel—the biogas (Holm-Nielsen et al. 2009)—digestates are of great potential interest for use in agriculture since they provide essential plant nutrient elements together with easily decomposable organic substrates (Tambone et al. 2010, 2013; Makádi et al. 2012). However, their effect on soil fertility status can be highly variable depending on their chemical properties, nutrient content, and biochemical stability (Alburquerque et al. 2012; Insam et al. 2015). Digestates can also contain undesirable harmful compounds such as heavy metals (Kupper et al. 2014), organic pollutants (i.e., pesticides, phenols), antibiotics, as well as various pathogenic bacteria (e.g., *Salmonella* spp., *Listeria* spp., *Escherichia coli*), that once introduced into the soil–plant system can produce considerable ecotoxicological impacts upon soil microbial communities and their functions (Govasmark et al. 2011; Bonetta et al. 2014).

Soil microbial properties are sensitive and early indicators of soil responses to land-use change, external disturbance, and anthropogenic pressure (Kennedy and Smith 1995; Jangid et al. 2008). The assessment of their compositional shifts determined by amplicon sequencing or metagenome analysis has become widely used to investigate the impact of different management practices upon arable soils (Vestergaard et al. 2017). In addition, the combined DNA/RNA profiling allows gaining a deeper insight into compositional responses of either metabolically active (16S rRNA) or total (16S rRNA gene) soil microbial communities (Zwolinski 2007; Christen 2008; Delmont et al. 2011; Caporaso et al. 2012).

In order to investigate the short-term impact of two improved soil management practices (namely organic amendment with a solid anaerobic digestate, and no-tillage) on the fertility status of two Mediterranean woody crop systems, we carried out a 1-year field experiment in two orchard soils (olive, citrus grove) with contrasting texture (clay, sandy loam), carbonate content (non-calcareous, slightly calcareous), and pH (strongly acid, slightly alkaline). In a parallel finding, Badagliacca et al. (2020) have shown that in contrast to conventionally managed soils, either the incorporation of a solid anaerobic digestate or no-tillage selectively altered aggregate stability, microbial C-use efficiency, and soluble N pool dynamics with differences in magnitude and persistence strongly related to soil texture and carbonate content. Since physical, chemical, and biological soil responses are strongly interrelated, the present study focused on assessing immediate (2 days) and short-term (7 and 18 weeks) compositional shifts in the phylogenetic diversity of both the total and the metabolically active prokaryotic communities as determined by the same treatments in the olive and citrus orchard soils. To this aim, we employed a culture-independent approach based on soil DNA and RNA direct extraction method followed by high-throughput next-generation sequencing of both 16S rRNA gene template (16S rDNA) and reverse-transcribed 16S rRNA targets (16S rRNA). The phylogenetic diversity of soil bacterial and selected archaeal communities were observed, and the α-diversity indices calculated. The molecular characterization of the indigenous bacterial community of a solid anaerobic digestate was also investigated, as well as changes in selected chemical soil variables. Findings from organically managed and no-tilled plots were compared to those from conventionally tilled ones. The hypotheses assumed for this study were (1) active and total prokaryotic communities differ significantly in their compositional response to improved soil management practices (*H1*), and (2) field addition of solid anaerobic digestate brings about immediate compositional shifts of soil prokaryotic community composition whose magnitude and persistence are primarily controlled by soil properties (*H2*). Combined assessment of prokaryotic diversity and activity was extended over time (from days to months) to consider temporal dynamics beside management-related responses.

Materials and methods

Digestate

Solid anaerobic digestate was provided by a local medium-scale biogas producing plant (< 1 MW) operating under mesophilic conditions (T ~ 40 °C). The biogas plant was supplied with dairy cattle slurry (70%), solid wastes from citrus and olive processing plants, pruning materials, maize silage, crop residues (20%), and milk serum (10%), as in Badagliacca et al. (2020). The resulting digestate was mechanically separated into the aqueous fraction (named liquor), which was discarded, and the solid fraction, which was collected and characterized (Table 1) according to Tambone et al. (2010) and Bonetta et al. (2014) before being used in the present

Table 1 Chemical, biochemical, and microbiological properties of the solid fraction of the anaerobic digestate. Values are means ± SD ($n = 3$) expressed on a dry matter basis

Parameter	Value
Chemical analyses	
pH[a]	8.77 ± 0.01
EC (dS m^{-1} at 25 °C) [b]	2.14 ± 0.01
Dry matter (% fresh weight)	18.0 ± 0.49
Ash (%)	14.4 ± 0.16
Volatile solids (%)	85.6 ± 0.16
Tot-C (g kg^{-1})	389.6 ± 0.8
Tot-N (g kg^{-1})	16.02 ± 0.70
C/N	24.3 ± 1.5
NH_4^+-N (g kg^{-1})	5.59 ± 0.47
NH_4^+-N (% Tot-N)	34.9
NO_3^--N (g kg^{-1})	0.034 ± 0.002
Tot-polyphenols (mg g^{-1})[c]	1.62 ± 0.05
P (g kg^{-1})	1.24
K (g kg^{-1})	2.25
S (g kg^{-1})	0.218
Ca (g kg^{-1})	0.971
Mg (g kg^{-1})	0.789
Cl (g kg^{-1})	0.180
Fe (mg kg^{-1})	55.0
Mn (mg kg^{-1})	53.0
B (mg kg^{-1})	9.0
Cd (mg kg^{-1})	< 0.01
Cr_{VI} (mg kg^{-1})	0.97
Pb (mg kg^{-1})	0.07
Ni (mg kg^{-1})	1.26
Hg (mg kg^{-1})	< 0.1
Cu (mg kg^{-1})	1.92
Zn (mg kg^{-1})	25.2
Biochemical analyses	
Dehydrogenase activity (μg INTF g^{-1} 2 h^{-1})	37.93 ± 0.49
FDA-hydrolase activity (μg fluorescein g^{-1} h^{-1})	255.16 ± 5.16
Alkaline phosphatase activity (μg *p*-NP g^{-1} h^{-1})	328.00 ± 17.66
Microbiological analyses	
Salmonella spp. (MPN 25 g^{-1})	Absent
Escherichia coli (CFU g^{-1})	< 10^3

[a]In a biomass:water (3:50, w/v) mixture

[b]In a biomass:water (1:10, w/v) mixture

[c]As gallic acid, determined by Folin-Ciocalteu's reagent method

experiment. Briefly, the pH and the electrical conductivity (EC) were measured in a biomass:water (3:50 and 1:10, w/v, respectively) mixture; the ash content was determined after combustion at 550 °C for 8 h; total organic C and N were measured by an automatic elemental analyzer CN628 LECO (LECO Corporation, MI, USA); NH_4^+-N and NO_3^--N content were determined colorimetrically in biomass:2 M KCl solution (1:10, v/v) extracts by using a Flow Injection Analysis System (FIAS 400 PerkinElmer, Inc., CT, USA) equipped with an AS90 Autosampler (PerkinElmer) and linked to an UV/Vis spectrophotometer Lambda 25 (PerkinElmer); total P, K, S, Ca, Mg, Cl, F, Mn, B. Cd, Cr, Pb, Ni, Hg, Cu, and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS ICAP-Q Thermo Scientific, CA) after microwave (Ethos up, Milestones srl, I) acid digestion with HNO_3/H_2O_2 (7:1 v/v); enzymatic activities (namely alkaline phosphatase, dehydrogenase, and hydrolysis of fluorescein diacetate) were determined colorimetrically as described by Dick et al. (1996). All analyses were carried out in triplicate.

Study sites

The field experiment was established during the 2016/2017 growing season in two orchard sites (an olive and a citrus grove) located within the Calabrian region (Southern Italy) with soils that exhibited contrasting texture, carbonate content, and pH (Supplementary Fig. 1).

The olive (*Olea europaea* L. cv. *Carolea*; 70-year-old plants with a planting distance of 6 × 6 m) orchard is located in the area nearby Lamezia Terme (Catanzaro, 38° 58′ N, 16° 18′ E, 81 m above the sea level) in an area characterized by mild and rainy winters and relatively warm and dry summers. Mean annual rainfall and air temperature were, respectively, 1094 mm and + 14.3 °C (averages over the 1985–2015 period) (Arpacal 2018). Soil thermal and moisture regimes were thermic and udic (first 150 cm), respectively. The soil is classified as Typic Hapludalf fine, mixed thermic (Soil Survey Staff 2010), or Cutanic Profondic Luvisol (IUSS Working Group WRB 2006). The soil evolves over ancient conoids forming a terrace plane constituted of Pleistocene sands and brown-reddish conglomerates of metamorphic origin. Slope is less than 10% facing a W exposure. Soil depth is > 180 cm and the available water-holding capacity (AWC, available moisture between the field capacity and the wilting point) equals 180 mm. Drainage is good and permeability is moderately slow. The soil is an acid clayey soil (Table 2). Cultivation with olive trees was established in the mid-50 s, and ever since, the soil has been continuously cropped and periodically ploughed (till layer 0–20 cm).

The citrus (*Citrus sinensis* (L.) Osbeck cv. *Tarocco*; 30-year-old plants with a planting distance of 4 × 4 m) orchard is located near Locri (Reggio Calabria, 38° 13′ N, 16° 14′ E, 12 m above the sea level) in an area characterized by mild rainy winters and warm and arid summers. Mean annual rainfall and air temperature were, respectively, 792 mm and + 18.3 °C (averages over the 1988–2015 period) (Arpacal 2018). Soil thermal and moisture regimes were thermic and xeric (first 150 cm), respectively. The soil is classified as Typic Xerofluvent (Soil Survey Staff 2010) or

Table 2 Main physical and chemical properties of tested soils from the two study sites. Values are means ± SD (*n* = 3) expressed on a dry weight basis

Soil variable	Study site	
	Olive orchard	Citrus orchard
Coarse sand (%)	6.6 ± 0.1	23.7 ± 0.7
Fine sand (%)	12.3 ± 0.3	34.0 ± 0.8
Coarse silt (%)	13.6 ± 0.3	17.3 ± 0.3
Fine silt (%)	22.5 ± 0.3	12.5 ± 0.3
Clay (%)	45.0 ± 0.8	12.5 ± 0.6
Texture (according to USDA)	Clay	Sandy loam
Bulk density (g cm^{-3})	1.48 ± 0.02	1.22 ± 0.14
Structural stability index (%)	73.9 ± 7.5	44.7 ± 11.1
pH_{CaCl2}	5.44 ± 0.11	7.46 ± 0.12
$EC_{1:2}$ (dS m^{-1})	0.170 ± 0.013	0.210 ± 0.087
Total $CaCO_3$ (g kg^{-1})	0	22.5 ± 3.0
Active $CaCO_3$ (g kg^{-1})	0	6.9 ± 0.1
CEC ($cmol_+$ kg^{-1})	51.9 ± 2.4	36.1 ± 1.2
Ca/Mg	4.42	10.15
Mg/K	0.94	6.28
Base saturation (%)	31 ± 3	100 ± 2
C_{org} (g kg^{-1})	21.30 ± 3.24	13.74 ± 0.15
N_t (g kg^{-1})	2.03 ± 0.29	1.03 ± 0.05
C/N	10.51 ± 0.35	13.34 ± 0.66
Exchangeable NH_4^+-N (mg kg^{-1})	3.2 ± 0.2	5.1 ± 1.0
NO_3^--N (mg kg^{-1})	2.8 ± 2.0	2.2 ± 1.3
Olsen-P (mg kg^{-1})	22.9 ± 2.2	20.4 ± 2.1

Fluvi Calcaric Cambisol (IUSS Working Group WRB 2006). The soil evolves over Holocene alluvial deposits from the nearest river Gerace. Soil depth is > 180 cm and the available water-holding capacity (AWC) equals 170 mm. Drainage is good and permeability is moderately high. The soil is a slightly calcareous sandy loam soil (Table 2) and has been cultivated with orange trees for the past 30 years and conventionally tilled to the depth of 20 cm.

Management and inputs into the soils at each site were consistent over the past 70 years (olive orchard) or 30 years (citrus orchard) and were thus considered to be uniform in terms of fertility.

Experimental design and soil treatments

At each site, the experimental set up consisted of field plots (75 m × 18 m each) arranged in a randomized complete block design, with three replications, in order to compare the following three treatments: (1) no-tillage (NT) where weeds were controlled by mechanical mowing and their biomasses was left on the soil surface as a residue mulch; (2) conventional tillage (TILL) consisting of inter-row harrowing (~ 20 cm) followed by slight rolling; (3) digestate incorporation (DIG), which comprised the TILL treatment combined with soil incorporation of a solid anaerobic digestate at a rate of 30 Mg ha^{-1}. This dose, established by considering digestate dosages commonly used in agriculture, is also similar to that used by other authors in C and N mineralization field experiments using organic conditioners (Barra Caracciolo et al. 2015; Fernández-Bayo et al. 2017). According to traditional practices, field plots were fertilized with 400 kg ha^{-1} of a 20 N-10P_2O_5-10K_2O chemical fertilizer supplying 80 kg N ha^{-1}, 18 kg P ha^{-1}, and 34 kg K ha^{-1}. Since the annual rainfall was markedly different between the two study sites, the olive orchard was managed under rainfed conditions. In contrast, the citrus orchard was drip irrigated in order to supply 200–300 mm water during the dry summer period to avoid water stress and maintain size, quality, and yield of fruit. The drip tube was located along the row under the tree skirt to provide a wetted band of approximately 0.5 m along each side of the trees, which did not overlay with the sampling area.

Soil sampling

Soil samples were collected during the 2016/2017 growing season 6 days before (Pre-treat, early May) and then 2 days (T1, May), 7 weeks (T2, late June), and 18 weeks (T3, mid-September) after the treatment application. Three individual non-rhizosphere soil cores (approx. 200 g each) were surface collected (Ap horizon, 0–20 cm soil layer) from the middle of each of the three inter-row space, to minimize any border and plant effect, and then thoroughly mixed to form a unique composite sample. Three composite samples (each from 9 individual inter-row soil cores) were taken per treatment. To sum up, nine composite samples were collected at each sampling time per study site, thus producing an overall amount of 72 composite soil samples (3 treatments × 3 replicates × 4 sampling times × 2 sites). Soon after the sampling, each soil sample was split in two aliquots: a representative amount (approx. 50 g) was sieved at < 2-mm particle size, and immediately frozen on-site using liquid nitrogen and then stored at −80 °C on return to the laboratory before being processed for DNA/RNA extraction. The remaining field-moist aliquot was stored in clean plastic bags and then in the laboratory air-dried, sieved at < 2-mm particle size, and stored at room temperature before physical and chemical characterization. Care was taken to keep the field equipment sterile and prevent cross-contamination of soil samples during and after collection. Sampling was limited to 20-cm topsoil where microbial numbers are higher and rapidly responsive to environmental impacts (Joergensen and Emmerling 2006). As for the sampling strategy, basic guidelines recommended by Vestergaard et al. (2017) were carefully followed.

Chemical analysis

Soil chemical properties (i.e., pH, $EC_{1:2}$, C_{org}, N_t) were determined using the standard methods recommended by the Soil Science Society of America (Sparks 1996). Briefly, soil pH was measured potentiometrically in a soil:0.01 M $CaCl_2$ solution (1:2.5, w/v) mixture, while soil electrical conductivity (EC) was measured in a soil:water (1:2, w/v) mixture. Total organic C (C_{org}) and N (N_t) were determined in finely ground (< 0.5 mm) soil samples by an automatic elemental analyzer LECO CN628 (LECO Corporation). K_2SO_4-extractable C (Extr-C) and total soluble N (TSN) were determined, respectively, in soil:0.5 M K_2SO_4 solution (1:4, w/v) and soil:2.0 M KCl solution (1:10, w/v) extracts by using an automatic elemental analyzer TOC-L CSH Shimadzu (Shimadzu Corporation, Tokyo, J) equipped with a TMN-L module for total N determination and an ASI-L autosampler unit (Shimadzu Corporation). Extr-C and TSN are here considered, respectively, as total soil C and N soluble pools.

Molecular analysis: DNA/RNA extraction, PCR amplification, sequencing

For total community analysis, genomic DNA was extracted from 250 mg of field-moist soil by using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The successful extraction was confirmed by 1.2% agarose gel electrophoresis. Soil DNA extracts were stored at −20 °C before further molecular analysis. For metabolically active community analysis, total soil RNA was extracted from 2.0 g of field-moist soil by using the RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories). In order to remove any contaminating DNA, soil-extracted RNA was first treated with TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After the DNase treatment, extracted 16S rRNA was PCR checked for possible DNA contamination. Afterwards, approximately 1 µg of soil-extracted RNA was reverse transcribed by using the Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit (Life Technologies) for reverse RNA transcriptase according to the manufacturer's protocol. Template RNA was substituted with sterile dd-H_2O in the PCR-negative control sample. Reverse-transcribed soil-extracted RNA was stored at −20 °C before further molecular analysis.

The V3–V4 region of the 16S rDNA (total prokaryotic community) and reverse-transcribed 16S rRNA (active prokaryotic community) templates was amplified using the Illumina barcoded primer pair Bakt341F/Bakt805R (Klindworth et al. 2013) using a Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, D). The PCR reaction mix (50 µl) contained 40 ng of template DNA, 1X Invitrogen™ SuperFi™ Buffer plus and 1X Invitrogen™ SuperFi™ GC Enhancer + 7.5 mM $MgCl_2$ (Thermo Fisher Scientific, Carlsbad, CA, USA), 0.02 Units μl^{-1} of Invitrogen™ Platinum™ SuperFi™ DNA Polymerase (Thermo Fisher Scientific), 0.4 µM of each primer, 0.2 mM of each dNTP. PCR running conditions were 3-min denaturation at 95 °C, followed by 30 sequential cycles each consisting of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, followed by a final extension step at 72 °C for 7 min. Template DNA/cDNA was substituted with sterile dd-H_2O in the PCR-negative control sample. PCR products (amplicon size was ~ 550 bp) were resolved on a 2% agarose gel, and then purified using a GENECLEAN® SPIN Kit (QBiogene, Inc., Carlsbad, CA, USA). Purified amplicons were quantified by an Invitrogen™ Qubit™ 2.0 Fluorometer (Thermo Fisher Scientific). Purified amplicons were used for library preparation and sequencing, according to the Illumina 16S Metagenomic Sequencing Library Preparation guide (downloaded from https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Paired-end sequencing (2 × 300 bp) was carried out by using an Illumina MiSeq™ platform (Illumina Inc., San Diego, CA, USA).

Statistics and sequence analysis

Chemical and biochemical data

Soil chemical and biochemical data, reported as mean values ($n = 3$), were expressed on a dry weight (dw) basis (105 °C, 24 h). They were first tested for deviation from normality (Kolmogorov–Smirnov test) and homogeneity of within-group variances (Levene's test). After running a two-way analysis of variance (ANOVA, soil management x time) to check any significant effect of soil management, time, and their interaction on the variability of the data (the block effect in the experimental design was found to be not significant at $P < 0.05$), multiple pairwise comparisons of means was done by Tukey's HSD (honestly significant difference) test at $P < 0.05$ level of significance. Statistical data processing was done by using SYSTAT 13.0 of SYSTAT Software Inc. (Erkrath, D).

Molecular data

Paired reads were assembled, quality-filtered, and analyzed using the SEED 2.0.3 software (Větrovský and Baldrian 2013) implemented with UCHIME, UPARSE, and USEARCH software (Edgar 2010, 2013; Edgar et al. 2011) at the time of analysis. Briefly, chimeric sequences were detected using the UCHIME algorithm and removed from the dataset. Sequences were then clustered into operational taxonomic units (OTUs) at a 97% sequence identity threshold using the UPARSE algorithm and then consensus sequences

were constructed for all clusters (Větrovský and Baldrian 2013). Global single and doubletons were excluded from further analyses. Identification and taxonomic assignment were done using representative sequences retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) and Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) at a 10^{-4} E value threshold. Sequences identified other than bacteria were discarded. Phylogenetic assignment to bacterial phyla and class levels was based on best hits, by dividing the number of sequences belonging to each phylogenetic group by the total number of sequences in the given sample.

Diversity analysis of OTUs was performed on resampled datasets with the same number of sequences randomly selected from all samples (20,000 sequences). The rarefaction curves were created by randomly re-sampling the pool of OTUs obtained at class level considering either the total (from 16S rDNA templates) or metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic taxa surveyed both in the olive and in citrus cropped soil across different treatments taken at the final sampling time (T3, 18 weeks) (Supplementary Fig. 2). OTU distribution among samples was also analyzed in SEED 2.0.3 software used to calculate the Shannon diversity index (H′), the richness (S), the evenness (E), and the Chao1 index, which were used to estimate the α-diversity of the bacterial communities.

PAST 3.06 (Hammer et al. 2001) and R Statistical Environment (R development Core Team 2008) were used for all statistical analysis. Diversity indices were then statistically analyzed by two-way ANOVA (soil management × time) to check any significant effect of soil management, sampling time, and their interaction on the variability of the data. OTUs with > 0.1% relative abundance were used to evaluate differences in β-diversity.

Principal coordinate analysis (PCoA) and PERMANOVA test were conducted based on Bray–Curtis similarity distance to determine the distribution of diversity and statistical significance of β-diversity, respectively. Correlations between β-diversity and physicochemical soil variables were determined by Canonical Corresponding Analysis (CCA) followed by Mantel test. The forward selection was based on Monte Carlo permutation tests (permutations D 999) (Anderson et al. 2003). The ordination in the X- and Y-axes and the length of the corresponding arrows indicated the importance of soil chemical variables in explaining the taxa distribution across communities. Similarity percentage (SIMPER) test was run to identify what OTUs contributed the most to discriminate the soil bacterial community during the trial. The co-occurrences of the 500 most abundant OTUs in prokaryotic communities of each treatment for both sites were analyzed by calculating Spearman's rank coefficients (P) using the R package "Hmisc" (Harrell 2008). Subsequently, those significant (FDR adjusted P value < 0.01) and robust ($P \geq 0.6$) correlations between OTUs were exported as a GML format network file using R package igraph (Csardi and Nepusz 2006). Network visualization was conducted using the Fruchterman-Reingold layout of the interactive platform Gephi version 0.9.2. Possible keystone genera were those that demonstrated high betweenness centrality values (Vick-Majors et al. 2014). The modular structure of the community was evaluated via the modularity index (Lambiotte et al. 2014). Finally, the Venn diagram was constructed to identify unique and shared OTUs between all digestate-treated soils and the solid anaerobic digestate.

All Illumina datasets were submitted to the European Nucleotide Archive (ENA) under the study accession number PRJEB44243.

Results

Soil chemical properties

The two orchard soils showed contrasting physical and chemical properties (Table 2). The olive cropped soil was characterized by strong acidic conditions, absence of carbonates, unbalanced Ca/Mg and Mg/K ratios, and thus low availability of calcium and magnesium, potential toxicity due to soluble aluminum, iron, and manganese, high clay content, poor aeration, slow drainage, and high bulk density. These physical and chemical conditions are fairly restrictive to olive tree cultivation and reaching high-quality crop yield can be severely constrained. On the contrary, the citrus cropped soil showed a slightly alkaline pH, low content of carbonates, low availability of phosphorus and iron, a sandy loam texture, stoichiometrically balanced Ca/Mg and Mg/K ratios, excessive drainage and high aeration, which exposes the native soil organic matter to a rapid mineralization process. In this soil, crop yield and production may be constrained by nutrient leaching and low availability of nutrient elements such as phosphorus, iron, manganese, and zinc.

In both soils, no significant effect (two-way ANOVA, management × time) was observed on soil pH which remained practically unaffected across the whole experimental period, exception being the DIG treatment at T1 (2 days after the start of the trial) in the acid olive orchard soil where a marked but transient increase was observed (Table 3). Conversely, the electrical conductivity (EC) was significantly affected by the experimental factors (two-way ANOVA; Table 3). The DIG-treated plots exhibited a marked increase in the EC soon after the amendment and a long-lasting effect was appreciable even though with a different trend depending on the soil type. In contrast, the TILL treatment exhibited no effect in the citrus cropped soil, whereas in the olive orchard soil, it raised immediately the EC by approximately 50% with respect to the initial (Pretreat) level (Table 3). Finally, no-tillage did not produce any

Table 3 Changes in chemical properties (mean ± SD, *n* = *3*) in the olive and citrus cropped soils under different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 growing season. Different letters indicate significant differences within each sampling time (Tukey's HSD at $P < 0.05$). Significant effects due to soil management (M), time (T), and their interaction are presented as *F*-values and level of significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) estimated by two-way ANOVA (management × time)

Site	Soil variable	Sampling time											
		Pre-treat			T1			T2			T3		
		NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG
Olive orchard	pH_{CaCl2}	5.3 ± 0.1	5.4 ± 0.1	5.4 ± 0.2	5.3 ± 0.1^{b}	5.2 ± 0.2^{b}	5.8 ± 0.1^{a}	5.1 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	5.2 ± 0.2	5.1 ± 0.1	5.4 ± 0.2
	$EC_{1:2}$ [#]	163 ± 24	173 ± 40	159 ± 26	164 ± 23^{c}	264 ± 42^{b}	485 ± 18^{a}	199 ± 16^{c}	256 ± 30^{b}	390 ± 20^{a}	254 ± 31^{b}	246 ± 21^{b}	347 ± 23^{a}
		M: $F_{2,24} = 61.20$***; T: $F_{3,24} = 51.76$***; M × T: $F_{6,24} = 15.47$***											
	C_{org} [†]	20.2 ± 2.1	19.6 ± 0.9	22.0 ± 2.8	20.3 ± 1.4^{b}	23.5 ± 2.5^{b}	28.8 ± 1.3^{a}	19.7 ± 1.4^{b}	21.1 ± 2.4^{b}	26.4 ± 1.7^{a}	21.7 ± 1.0^{b}	19.2 ± 1.0^{c}	25.7 ± 2.3^{a}
		M: $F_{2,24} = 4.19$*; T: $F_{3,24} = 2.96$*											
	Nt[†]	1.8 ± 0.1	1.8 ± 0.1	1.9 ± 0.2	1.9 ± 0.1^{b}	1.9 ± 0.1^{b}	2.6 ± 0.1^{a}	1.8 ± 0.2^{b}	1.8 ± 0.1^{b}	2.3 ± 0.1^{a}	2.2 ± 0.1^{ab}	1.9 ± 0.1^{b}	2.4 ± 0.3^{a}
		M: $F_{2,24} = 3.86$*											
	Extr-C[$]	206.5 ± 34.1	175.6 ± 34.1	205.2 ± 20.6	204.1 ± 20.0^{b}	169.0 ± 12.7^{b}	286.0 ± 24.5^{a}	252.5 ± 50.7^{b}	237.2 ± 32.2^{b}	370.0 ± 27.2^{a}	245.7 ± 1.6	209.7 ± 49.8	259.1 ± 16.5
		M: $F_{2,24} = 8.60$***; T: $F_{3,24} = 16.57$***; M × T: $F_{6,24} = 2.45$*											
	TSN[$]	31.8 ± 1.0	30.8 ± 3.1	31.9 ± 2.5	49.1 ± 8.4^{b}	44.7 ± 1.2^{b}	115.1 ± 26.8^{a}	32.6 ± 5.9^{b}	41.1 ± 4.3^{b}	85.5 ± 1.7^{a}	40.6 ± 6.6^{b}	48.6 ± 5.9^{b}	65.5 ± 6.7^{a}
		M: $F_{2,24} = 22.94$***; T: $F_{3,24} = 71.02$***; M × T: $F_{6,24} = 9.26$***											
Citrus orchard	pH_{CaCl2}	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.2 ± 0.1	7.1 ± 0.1	7.2 ± 0.2	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.4 ± 0.1
	$EC_{1:2}$ [#]	234 ± 25	236 ± 17	237 ± 11	260 ± 22^{b}	256 ± 10^{b}	386 ± 51^{a}	230 ± 20^{b}	259 ± 21^{b}	396 ± 61^{a}	263 ± 36^{b}	293 ± 22^{ab}	368 ± 51^{a}
		M: $F_{2,24} = 35.52$***; T: $F_{3,24} = 12.15$***; M × T: $F_{6,24} = 5.87$***											
	C_{org} [†]	13.5 ± 1.0	13.0 ± 0.5	13.6 ± 1.7	14.0 ± 0.5^{b}	14.0 ± 0.6^{b}	16.2 ± 1.2^{a}	14.4 ± 0.8^{b}	13.9 ± 0.6^{b}	15.9 ± 0.5^{a}	13.7 ± 1.2^{b}	13.6 ± 1.0^{b}	16.3 ± 0.5^{a}
		M: $F_{2,24} = 3.51$*; T: $F_{3,24} = 3.46$*											
	Nt[†]	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1^{b}	1.3 ± 0.1^{b}	1.6 ± 0.1^{a}	1.3 ± 0.1^{b}	1.2 ± 0.1^{b}	1.6 ± 0.1^{a}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.6 ± 0.1^{a}
		M: $F_{2,24} = 3.62$*; T: $F_{3,24} = 9.31$***											
	Extr-C[$]	136.9 ± 20.3	133.4 ± 8.9	130.5 ± 23.7	133.2 ± 18.3^{b}	141.7 ± 18.1^{b}	180.6 ± 14.4^{a}	156.5 ± 21.4^{b}	233.7 ± 34.6^{a}	215.6 ± 28.9^{a}	196.6 ± 20.2^{b}	245.0 ± 7.1^{a}	266.0 ± 17.9^{a}
		M: $F_{2,24} = 12.27$***; T: $F_{3,24} = 40.95$***; M × T: $F_{6,24} = 3.13$*											
	TSN[$]	24.9 ± 1.3	25.4 ± 1.2	26.9 ± 1.6	28.0 ± 1.6^{b}	26.5 ± 0.9^{b}	48.1 ± 3.6^{a}	24.5 ± 1.9^{c}	38.7 ± 2.4^{b}	51.5 ± 6.0^{a}	30.4 ± 1.4^{b}	28.2 ± 0.6^{b}	37.9 ± 1.7^{a}
		M: $F_{2,24} = 19.14$***; T: $F_{3,24} = 191.70$***; M × T: $F_{6,24} = 6.34$***											

[#] $\mu S\ cm^{-1}$; [†] $g\ kg^{-1}$; [$] $mg\ kg^{-1}$

significant variation in EC at both the experimental sites, and only time-dependent variations were found.

As for C_{org} and N_t, slight non-significant time-dependent fluctuations were observed in the NT and TILL treatments. Whereas in DIG-treated plots, both variables immediately increased, respectively, to 28.8 g C kg^{-1} and 2.6 g N kg^{-1} in the olive and to 16.2 g C kg^{-1} and 1.6 g N kg^{-1} in the citrus grove soil, and this rise was appreciable over the whole experimental period (Table 3).

Likewise, Extr-C and TSN were markedly and immediately increased in DIG treatments with a long-lasting effect depending on the soil type (Table 3). As well as this, the tillage event (TILL) increased the values of both variables and this increase was more pronounced in the olive than in the citrus orchard soil. On the contrary, in NT treatments, both Extr-C and TSN only showed time-dependent variations at both sites (Table 3).

Illumina sequencing-derived dataset and phylogenetic diversity of prokaryotic taxa surveyed in tested soils and solid anaerobic digestate

A dataset of 1,313,240 high-quality 16S rRNA gene sequences (read length ranging from 150 to 480 bp) was produced by Illumina sequencing. Of these, 472,680 were from the olive cropped soil, 734,202 were from the citrus cropped soil, and 106,358 from the solid anaerobic digestate. All sequences were clustered at 97% nucleotide similarity, resulting in 11,328 OTUs. Despite increasing rapidly, the rarefaction curves did not reach the saturation level, suggesting that the taxonomic diversity was not fully exploited (Supplementary Fig. 2). This was particularly true for OTUs obtained from 16S rRNA targets of digestate-treated soil at the olive orchard site. However, it is not uncommon for rarefaction curves not to reach a plateau in soil bacterial communities, with both sequencing depth and a number of valid reads determining the OTUs obtained. The phylogenetic assignment analysis enabled the classification of ~ 74% sequences at the phylum level and ~ 65% sequences at the class level.

In the acid clayey (olive orchard) soil, the phylogenetic diversity of the total bacterial community (16S rDNA targets) was primarily distributed among 10 different phyla: Proteobacteria (46.00%), Actinobacteria (22.51%), Planctomycetes (9.18%), Bacteroidetes (5.71%), Acidobacteria (4.90%), Gemmatimonadetes (2.83%), Chloroflexi (2.27%), Verrucomicrobia (1.70%), Firmicutes (1.61%), and Nitrospira (0.92%) (Supplementary Fig. 3). They comprised a high number of bacterial classes, in particular: α-*Proteobacteria* (20.24%), *Actinobacteria* (16.22%), β-*Proteobacteria* (9.41%), γ-*Proteobacteria* (9.32%), *Planctomycetacia* (9.18%), δ-*Proteobacteria* (7.09%), together with less represented classes. Although the composition of the metabolically active soil bacterial community (16S rRNA targets) was largely similar (as for major taxa such as Proteobacteria, Actinobacteria, and Planctomycetes together with Verrucomicrobia), noticeable differences were found in the relative abundance of less represented taxa, which increased (Firmicutes, 2.68 *vs* 1.61%; *Bacilli* occurred twice as in the total community) or decreased (Bacteroidetes, 4.54 *vs* 5.71%; class *Flavobacteriia* was nearly half than in the total community; Acidobacteria, 4.00 *vs* 4.90%; with a contrasting variation of classes *Blastocatellia* and *Solibacteres*; Gemmatimonadetes, 1.46 *vs* 2.83%; class *Gemmatimonadetes* was nearly half as in the total community; Chloroflexi, 1.11 *vs* 2.27%; class *Thermomicrobia* was half as in the total community) (Supplementary Fig. 3). Finally, ammonia-oxidizing archaea belonging to the phylum Thaumarchaeota (class *Nitrososphaeria*) showed a relative abundance ranging from 0.40% (16S rDNA targets) to 0.26 (16S rRNA targets) (*data not shown*).

The same 10 phyla previously observed (16S rDNA) represented most of the total bacterial community of the slightly calcareous loamy sand (citrus orchard) soil, whereas considerable changes in the relative abundance were observed for the following taxa: Firmicutes (3.41%) and Verrucomicrobia (2.94%; represented by class *Verrucomicrobiae* and *Spartobacteria*) which were more abundant than Bacteroidetes (4.15%), Gemmatimonadetes (2.25%; class *Longimicrobia* newly detected), Acidobacteria (2.14%; classes *Blastocatellia* and *Solibacteres* here not detected), and Chloroflexi (1.92%; classes *Chloroflexia* and *Ktedonobacteria* newly detected) (Supplementary Fig. 4). Same as in the olive orchard soil, the most abundant surveyed bacterial classes were α-*Proteobacteria* (20.59%), *Actinobacteria* (16.39%), and *Planctomycetacia* (9.26%), whereas appreciable differences were observed in γ-*Proteobacteria* (11.24%, more abundant), β-*Proteobacteria* (7.63%, less abundant) and δ-*Proteobacteria* (6.08%, less abundant). The metabolically active and total soil bacterial communities primarily differed for the relative abundance of minor taxa. Increased abundances occurred in the following groups: Bacteroidetes (4.78 *vs* 4.15%, variations in *Chitinophagia*, *Cytophagia*, and *Flavobacteria*) and Firmicutes (5.29 *vs* 3.41%, marked increase of class *Bacilli*). Decreased abundances occurred in the following groups: Actinobacteria, (20.83 *vs* 23.43%, reduced *Thermoleophilia* and *Rubrobacteria*), Verrucomicrobia (2.60 *vs* 2.94%), Acidobacteria (1.62 *vs* 2.14%), Gemmatimonadetes (1.55 *vs* 2.25%, with opposite changes in *Gemmatimonadetes* and *Longimicrobia*), Chloroflexi (1.18 *vs* 1.92%, variations in *Anaerolineae*, *Chloroflexia*, *Ktedonobacteria*, *Thermomicrobia*), and Nitrospira (0.59 *vs* 1.06%, class *Nitrospira* half abundant as in the total community) (Supplementary Fig. 4). As

for 16S rDNA targets, members of the phylum Thaumarchaeota (class *Nitrososphaeria*) showed a relative abundance ranging from 0.39% (16S rDNA targets) to 0.21 (16S rRNA targets) (*data not shown*).

The total and the active bacterial communities from the solid anaerobic digestate showed a fairly contrasting phylogenetic diversity at either phylum or class level. The total bacterial community was dominated by Firmicutes (58.16%, represented by *Clostridia* 43.40%, *Bacilli* 6.28%, *Tissierellia* 4.96%, and *Limnochordia* 3.52%), Proteobacteria (18.30%, dominated by γ-*Proteobacteria* 13.57%), and Bacteroidetes (15.50%, represented by *Flavobacteriia* 9.98% and *Bacteroidia* 5.48%), which taken together made up ~ 92% of the surveyed phyla (Supplementary Fig. 5). Minor components included members of classes *Thermales* (Deinococcus-Thermus) (4.43%), *Actinobacteria* (Actinobacteria) (1.18%), and *Mollicutes* (Tenericutes) (0.79%) together with "*Candidatus* Bacteria" (0.5%). Conversely, the most represented taxa of the metabolically active bacterial community were Proteobacteria (32.13%, especially classes α-*Proteobacteria* and γ-*Proteobacteria* which showed values as high as 12.94 and 11.73%, respectively), Firmicutes (28.47%, *Clostridia* and *Bacilli*, mainly), Actinobacteria (14.63%), and Bacteroidetes (6.98%) (Supplementary Fig. 5). Moreover, members affiliated with classes *Planctomycetacia* (Planctomycetes), *Thermomicrobia* (Chloroflexi), and "*Candidatus* Bacteria" (1.33%) were fairly represented in the active but at very low relative abundances in the total bacterial community. Meanwhile, the following class members were found in the active but were absent from the total bacterial community: *Erysipelotrichia* (Firmicutes) (2.25%), *Coriobacteriia* (Actinobacteria) (3.47%), and *Chitinophagia* (Bacteroidetes) (1.70%). Finally, members of *Methanomicrobia* (a class of Euryarchaeota), which include anaerobic archaeal microorganisms involved in the biological production of methane, were surveyed either in the total (~ 5%) or in the metabolically active (1.8%) prokaryotic community (Supplementary Fig. 5).

The Venn diagram revealed that only 12 OTUs (0.5%) of the entire surveyed Prokarya (total and active) were in common between digestate-treated soils and the solid anaerobic digestate (Fig. 1). Furthermore, total and active prokaryotic communities shared 512 OTUs (19.9%) in DIG-treated soils, while only 136 OTUs (5.3%) in the solid digestate. Interestingly, digestate-treated soils and solid anaerobic digestate shared a number as low as 17 OTUs (about 0.7%) of the total prokaryotic community, and 293 OTUs (about 11%) of the active prokaryotic community. Finally, it is noteworthy that the majority of OTUs of both the total (262, corresponding to 65%) and the active (709, corresponding to 62%) prokaryotic communities found in the anaerobic digestate were not observed in the recipient soils.

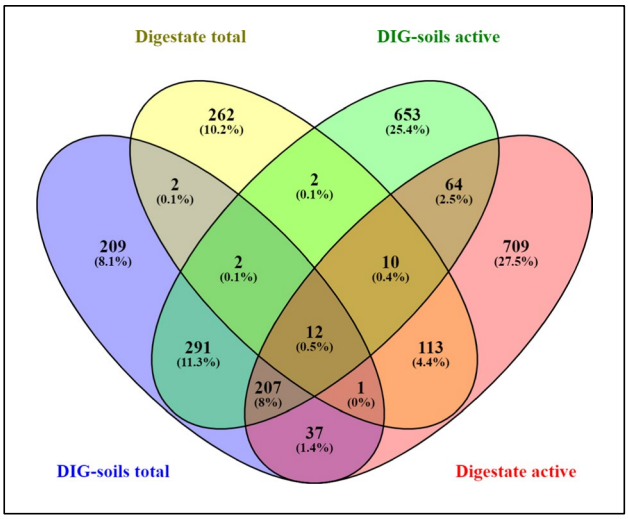
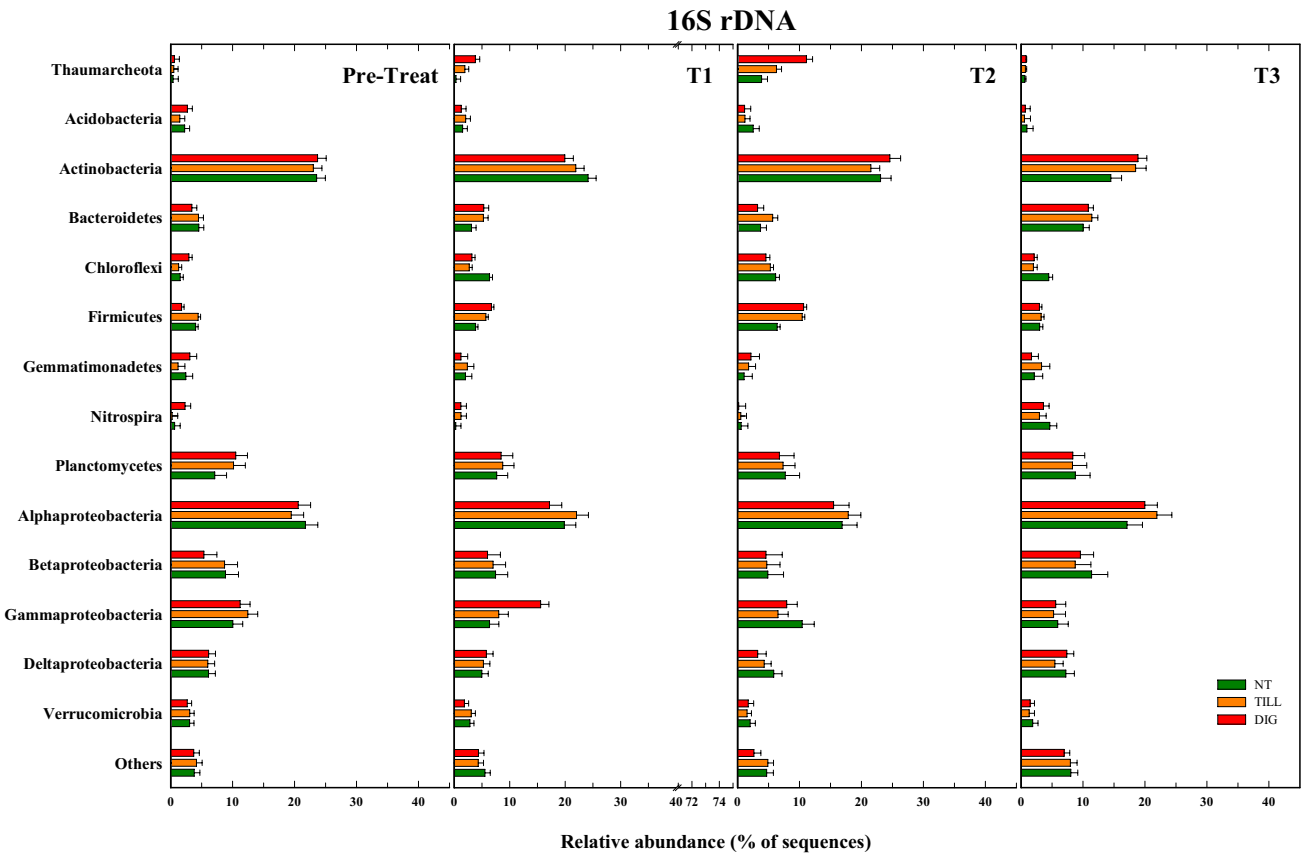


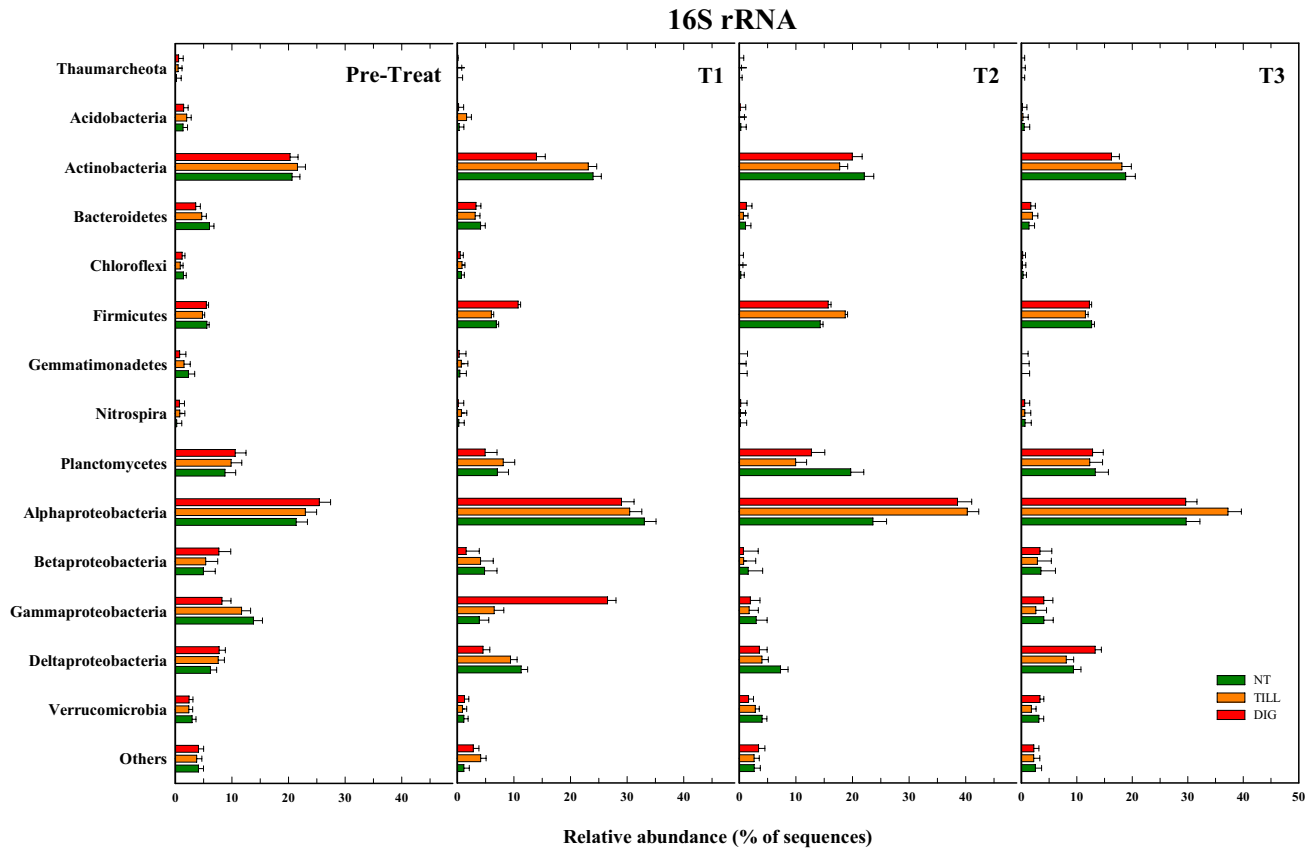
Fig. 1 Venn diagram showing exclusive and shared operational taxonomic units (OTUs) (at the 3% of evolutionary distance) between digestate-treated soils (all taken as a whole, DIG soils) and the solid anaerobic digestate (digestate) by considering both total (from 16S rDNA templates, left) and metabolically active (from reverse-transcribed 16S rRNA targets, right) prokaryotic soil communities

Compositional shifts and taxonomic diversity of total and active soil prokaryotic communities as affected by differing soil management systems and sampling time: the olive orchard soil

Major compositional changes in the total soil bacterial community (16S rDNA targets) were observed in the digestate-treated plots (Fig. 2A). Precisely, a considerable but short-lived increase in the relative abundance of Bacteroidetes (namely *Flavobacteriia*) and γ-*Proteobacteria* was found 2 days after the treatment (T1) accompanied by a marked decline of the other bacterial taxa, except Firmicutes, which remained unaltered. Indeed, this compositional change was fairly transient: an opposite trend was observed 7 weeks after the treatment (T2) and comparable abundance values among the three treatments were reached 18 weeks after the treatment (T3), with the exception of Acidobacteria (mainly *Acidobacteria* and *Solibacteres*) and α-*Proteobacteria*, which resulted less and more represented, respectively, than at the Pre-treat stage (Fig. 2A). Finally, even though the total bacterial community showed marked seasonal fluctuations in both NT and TILL treatments, selective changes were observed in the conventionally tilled plots at the last stage (T3), where the relative abundance of Acidobacteria (mainly *Acidobacteria*) increased, whereas that of Actinobacteria (*Actinobacteria*), Chloroflexi (*Thermomicrobia*, but not anaerobic *Anaerolineae*), γ-Proteobacteria, and Verrucomicrobia (*Spartobacteria*) declined (Fig. 2A).

The phylogenetic assignment of the metabolically active soil bacterial community (16S rRNA targets) of


16S rDNA
Pre-Treat
T1
T2
T3
Thaumarcheota
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospira
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Verrucomicrobia
Others
NT
TILL
DIG
Relative abundance (% of sequences)


16S rRNA
Pre-Treat
T1
T2
T3
Thaumarcheota
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospira
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Verrucomicrobia
Others
NT
TILL
DIG
Relative abundance (% of sequences)

◂**Fig. 2** Changes in relative abundances (mean ± SD, *n* = *3*) of total (**A**, from 16S rDNA templates) and metabolically active (**B**, from reverse-transcribed 16S rRNA targets) prokaryotic taxa in the olive orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Only are shown those taxa with average relative abundance > 1%

the digestate-treated soil partially confirmed the compositional shifts seen above, which involved fewer taxa (namely γ-*Proteobacteria*, Actinobacteria, Gemmatimonadetes, α-*Proteobacteria*, and Verrucomicrobia, but not Acidobacteria, Bacteroidetes, Chloroflexi, and Planctomycetes) with changes same in direction but less evident in magnitude (Fig. 2B). Besides time-dependent variations in the relative abundance of major metabolically active soil bacterial taxa registered in NT and TILL treatments, a minor relative abundance of Chloroflexi, Nitrospira, and Verrucomicrobia (*Spartobacteria*) was found in conventionally tilled plots at the last stage (T3) (Fig. 2B). Time-dependent variations were only found in the relative abundance of both total and metabolically active ammonia-oxidizing archaeal class *Nitrososphaeria* (phylum Thaumarchaeota) (Fig. 2A, B).

The two-way ANOVA showed that time significantly affected all the diversity indices, which markedly varied at both total and active soil prokaryotic community level across the experimental period (Table 4). Soil management significantly influenced the Chao1 index of the total community (with significant differences between tilled plots at T1 and T2 samplings), and on the evenness at level of the active community (Table 4). Soil management × time interaction had a significant effect on the Chao1 index (both total and active communities) as well as on taxa evenness (active community only) (Table 4).

CCA analysis and Mantel test were performed to correlate soil chemical variables with the prokaryotic communities' composition (Fig. 3). Output of the Mantel test indicated that a higher correlation was observed between soil edaphic properties and the total community compared to the active community. Total community was significantly correlated with pH, EC, TOC, and TN, while the active community was significantly correlated only with EC and TSN. The first two CCA axes explained 84.02% and 79.74% of the total variance, respectively, for the total and the metabolically active prokaryotic community (Fig. 3). Samples from the total prokaryotic community generally grouped according to their differing sampling times, whereas this was not so clear for the active community. Interestingly, samples from the DIG treatment at T1 (2 days after the treatment) clustered separately from all other samples, whatever the considered molecular pool (16S rDNA or 16S rRNA). For this treatment, distinctive groups were also noted at T2 (total) and T3 (active community).

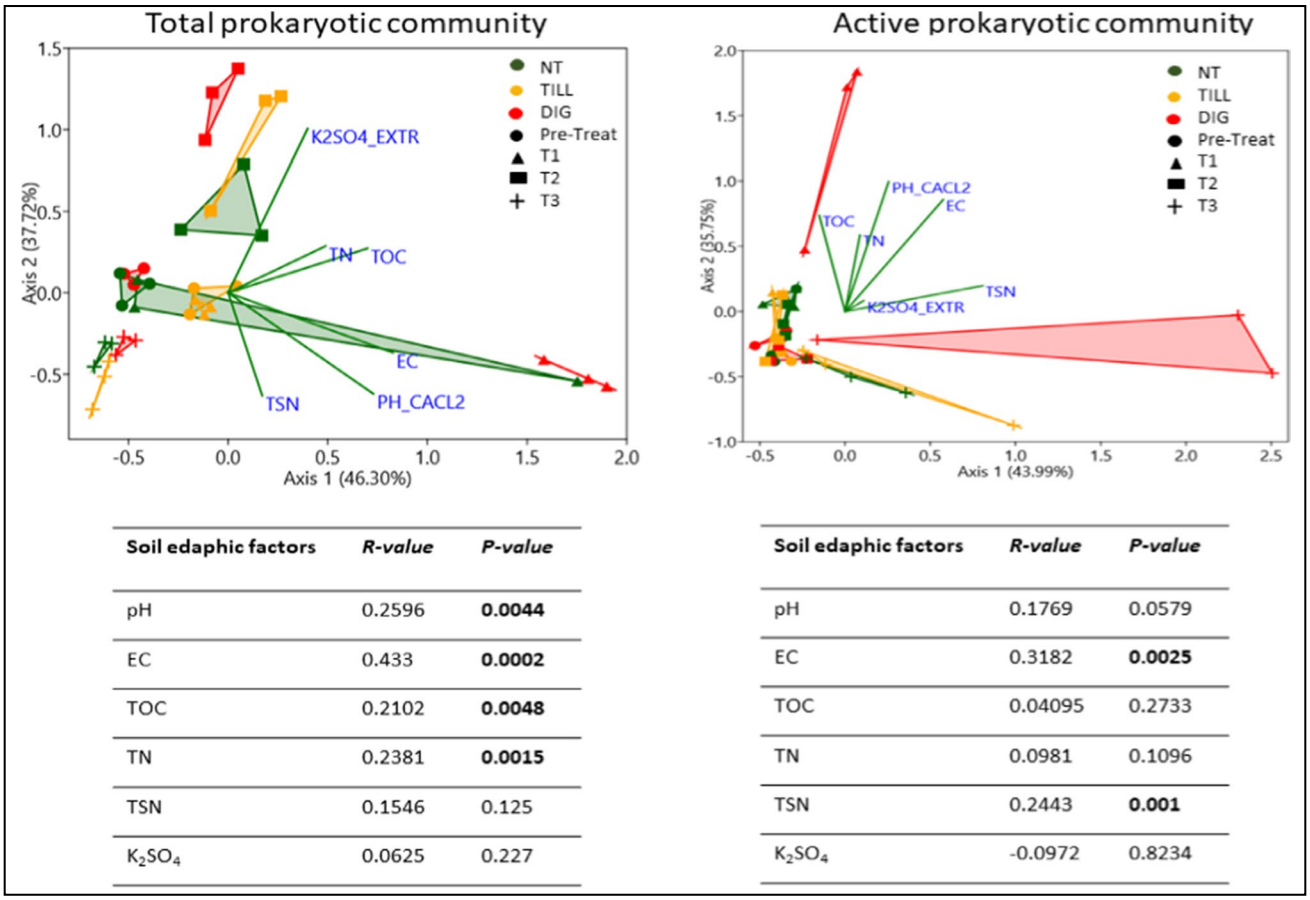
PCoA of Bray–Curtis distance was used to analyze the variation in the prokaryotic communities as affected by time and the management practices (Fig. 4). The significance level of variation was checked by PERMANOVA. The first two components, which accounted for 37.01% (16S rDNA) and 30.25% (16S rRNA) of the total explained variance, were used to visualize an ordination biplot PC1 *vs* PC2. Plots revealed that both total and active communities were clustered differently under different sampling times. PERMANOVA results confirmed a highly significant global effect of the sampling time (total community: $F = 5.56$, $P < 0.001$; active community: $F = 2.50$, $P < 0.001$). Management treatments did not exert a significant effect on the total prokaryotic community, whereas changes occurred in the active community, especially in DIG-treated plots which showed significant differences from that of NT ($P = 0.027$).

Co-occurrence patterns of the total and the metabolically active prokaryotic soil communities allowed to further characterize the selection effect of each management practice. The highest network complexity was found in the DIG, while the lowest one was observed in the NT treatment indicating that the network complexity gradually increased from NT to TILL and to DIG treatment (with an average degree of 5.076 in NT, 8.634 in TILL, and 23.25 in DIG) (Supplementary Fig. 6). The taxonomic composition of the networks differed among management practices, with more nodes belonged to Actinobacteria in DIG (22.92%) and TILL (23.26%), and to Actinobacteria in NT (28.21%). To sum up, Proteobacteria, Actinobacteria, and Acidobacteria resulted the most abundant bacterial taxa under all management practices.

The SIMPER test revealed which OTUs accounted for the majority of differences in the total and the active prokaryotic communities (Supplementary Table 1). In brief, in the digestate-treated soil, a marked, immediate, and short-lived increase of *Pseudomonas* spp. (γ-*Proteobacteria*) was observed both in the total and in the metabolically active community; *Dactylosporangium solaniradicis* (Actinobacteria) was particularly abundant in TILL samples at T2 and in DIG samples at T3; the filamentous Chloroflexi bacterium showed opposite changes in NT and TILL *vs* DIG samples; *Sphingomonas* spp. (α-*Proteobacteria*) strongly fluctuated in both tilled plots, especially after digestate addition; *Enterobacter* spp. (γ-*Proteobacteria*) showed time-dependent fluctuations particularly in NT and TILL treatments; *Bacterium Ellin 6099* (Acidobacteria) increased in both NT and TILL plots at the later stage. OTUs which contributed the most to discriminate the active prokaryotic community were *Arthrobacter* spp. (Actinobacteria) that was always highly abundant in digestate-treated soil; same as the two γ-proteobacterial *Pseudomonas* spp. and *Pseudomonas formosensis* observed before; in DIG plots, a transient increase after 7 weeks was found for the γ-proteobacterial OTU 49 (γ-*Proteobacteria* spp.), and a marked and late (18 weeks)

Table 4 Changes in the α-diversity indices of total (from 16S rDNA targets) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities surveyed in the olive orchard soil under different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 growing season. Different letters indicate significant differences within each sampling time (Tukey's HSD at $P<0.05$). Significant effects due to soil management (M), time (T), and their interaction are presented as F-values and level of significance (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) estimated by two-way ANOVA (management × time)

Molecular pool	Index	Sampling time											
		Pre-treat			T1			T2			T3		
		NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG
16S rDNA	H′	1.9 ± 0.3	2.1 ± 0.2	1.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.7	3.5 ± 0.1	3.0 ± 0.1	3.4 ± 0.2	3.5 ± 0.2	5.1 ± 0.1	4.1 ± 0.9	5.1 ± 0.1
		T: $F_{3,24}$ = 41. 2***											
	S	7.7 ± 2.3	8.7 ± 1.3	6.3 ± 0.3	60.3 ± 18.7	42.0 ± 6.1	57.3 ± 15.0	20.0 ± 0.6	32.2 ± 6.7	35.7 ± 8.0	200.0 ± 14.8	137.7 ± 64.8	219.7 ± 13.2
		T: $F_{3,24}$ = 43.35***											
	E	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	0.98 ± 0.05	0.95 ± 0.05	0.99 ± 0.01	0.99 ± 0.01	1.00 ± 0.00	0.99 ± 0.01	0.95 ± 0.00	0.96 ± 0.01	0.97 ± 0.02
		T: $F_{3,24}$ = 6.03**											
	Chao1	23 ± 2	38 ± 20	44 ± 11	850 ± 74^{a}	499 ± 83^{b}	533 ± 44^{b}	408 ± 58^{a}	210 ± 12^{b}	229 ± 15^{b}	665 ± 12	730 ± 13	399 ± 177
		M: $F_{2,24}$ = 8.43**; T: $F_{3,24}$ = 56.65***; M × T: $F_{6,24}$ = 3.60*											
16S rRNA	H′	2.6 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	3.0 ± 0.1	3.6 ± 0.6	3.7 ± 0.1	2.8 ± 0.2	2.5 ± 0.1	2.8 ± 0.1	3.1 ± 0.3^{a}	2.8 ± 0.3^{b}	2.1 ± 0.1^{b}
		T: $F_{3,24}$ = 11.37***											
	S	13.0 ± 1.0	11.0 ± 1.5	8.3 ± 0.7	20.7 ± 0.3^{b}	74.7 ± 55.2^{a}	78.7 ± 6.2^{a}	17.7 ± 3.3	12.7 ± 1.2	16.3 ± 1.4	24.3 ± 7.9	18.7 ± 6.2	8.5 ± 0.9
		T: $F_{3,24}$ = 5.40**											
	E	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.85 ± 0.02	1.00 ± 0.00	0.97 ± 0.03	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.99 ± 0.01
		M: $F_{2,24}$ = 15.72***; T: $F_{3,24}$ = 27.17***; M × T: $F_{6,24}$ = 19.89***											
	Chao1	39 ± 6^{b}	72 ± 6^{a}	51 ± 3^{ab}	376 ± 27^{a}	224 ± 7^{b}	305 ± 107^{b}	144 ± 25^{a}	126 ± 18^{a}	68 ± 5^{b}	40 ± 8^{b}	163 ± 23^{a}	86 ± 8^{b}
		T: $F_{3,24}$ = 31.24***; M × T: $F_{6,24}$ = 3.14*											



Soil edaphic factors	*R-value*	*P-value*
pH	0.2596	**0.0044**
EC	0.433	**0.0002**
TOC	0.2102	**0.0048**
TN	0.2381	**0.0015**
TSN	0.1546	0.125
K_2SO_4	0.0625	0.227

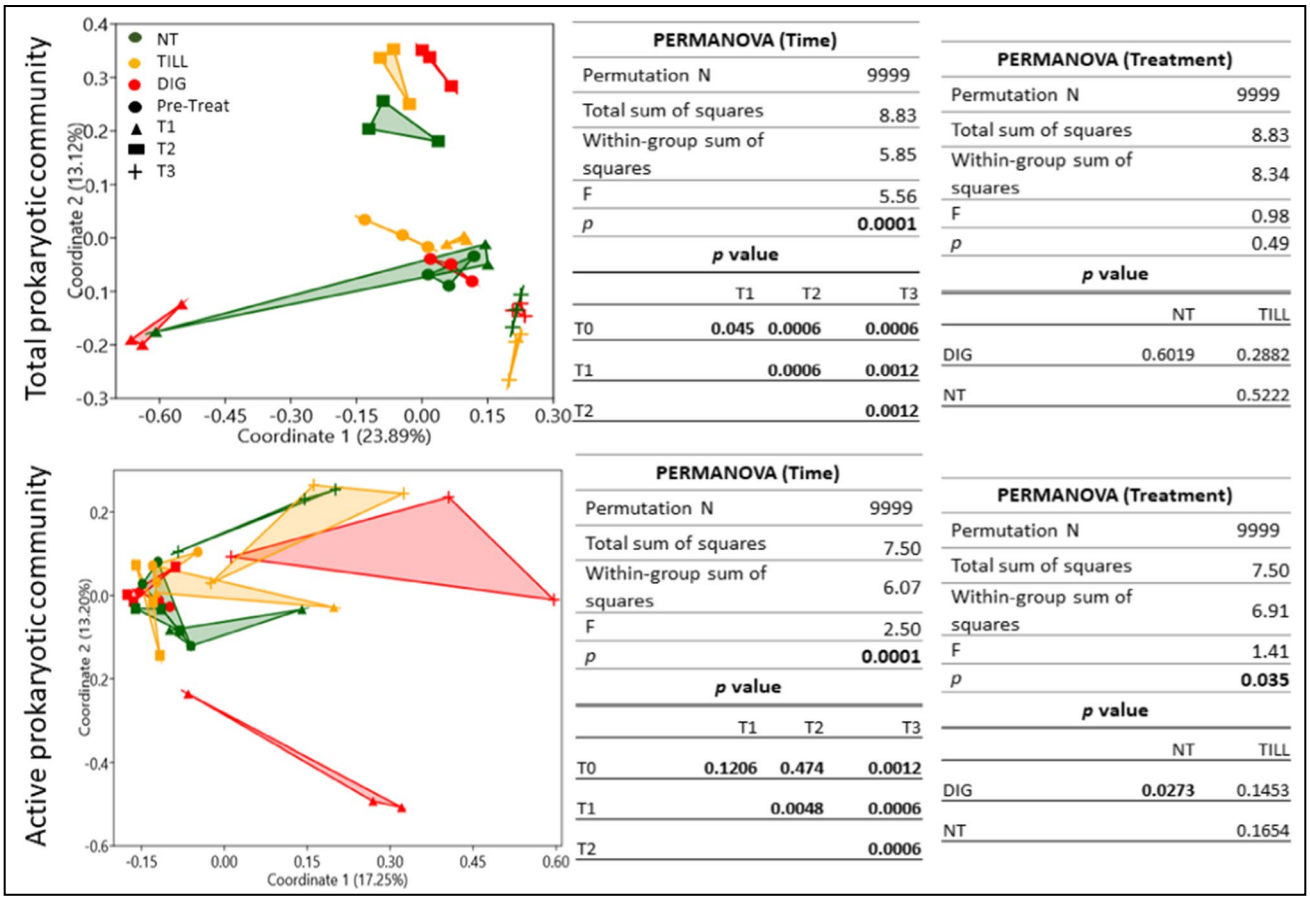
Soil edaphic factors	*R-value*	*P-value*
pH	0.1769	0.0579
EC	0.3182	**0.0025**
TOC	0.04095	0.2733
TN	0.0981	0.1096
TSN	0.2443	**0.001**
K_2SO_4	-0.0972	0.8234

Fig. 3 Canonical corresponding analysis based on Bray–Curtis similarity distance of OTUs with relative abundance > 0.1% of total (from 16S rDNA templates) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities of the olive orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Significant differences were detected by Mantel test (Pearson correlation coefficients)

increase for *Aquisphaera giovannonii* (Planctomycetes). The α-proteobacterial *Skermanella* spp. showed a contrasting trend between non-tilled (increase) and tilled soils (decline). Interestingly, *Escherichia* spp. (γ-*Proteobacteria*) was also detected as a member of the metabolically active community since before the start of the experiment and then declined in both tilled treatments (i.e. TILL, DIG), while remaining always detectable in the NT treatment. No discriminatory OTU belonging to archaeal taxa was found.

Compositional shifts and taxonomic diversity of total and active soil prokaryotic communities as affected by differing soil management systems and sampling time: the citrus orchard soil

As observed in the olive orchard soil, the most relevant compositional changes in the total soil prokaryotic community occurred in the digestate-treated soil, where an immediate and short-lived increase in the relative abundance of Proteobacteria (in particular γ-*Proteobacteria*) was observed at T1, followed by a significant decrease thereafter (Fig. 5A). Moreover, a slight, transient decline of Actinobacteria (primarily class *Thermoleophilia*, *Rubrobacteria*, and, although at a lesser degree, *Actinobacteria*), Gemmatimonadetes (*Gemmatimonadetes*), α-*Proteobacteria*, and Verrucomicrobia (*Spartobacteria*) was also observed shortly after the treatment event (Fig. 5A). Furthermore, conventional tillage, either without or with digestate addition, increased the relative abundance of Firmicutes (particularly *Bacilli*) and Bacteroidetes (namely *Chitinophagia*) 2 days and 7 weeks, respectively, after the beginning of the trial. Furthermore, an increase in the relative abundance of Chloroflexi (especially aerobic *Chloroflexia* and *Ktedonobacteria*) was recorded in the NT treatment throughout the observation period. The relative abundance of Nitrospira (class *Nitrospira*) oppositely varied in TILL (increase) and DIG (decline) plots at intermediate sampling times. Finally, only time-dependent changes were observed in no-tilled soil. At the final stage



PERMANOVA (Time)

Permutation N	9999
Total sum of squares	8.83
Within-group sum of squares	5.85
F	5.56
p	**0.0001**

***p* value**

	T1	T2	T3
T0	**0.045**	**0.0006**	**0.0006**
T1		**0.0006**	**0.0012**
T2			**0.0012**

PERMANOVA (Treatment)

Permutation N	9999
Total sum of squares	8.83
Within-group sum of squares	8.34
F	0.98
p	0.49

***p* value**

	NT	TILL
DIG	0.6019	0.2882
NT		0.5222

PERMANOVA (Time)

Permutation N	9999
Total sum of squares	7.50
Within-group sum of squares	6.07
F	2.50
p	**0.0001**

***p* value**

	T1	T2	T3
T0	**0.1206**	**0.474**	**0.0012**
T1		**0.0048**	**0.0006**
T2			**0.0006**

PERMANOVA (Treatment)

Permutation N	9999
Total sum of squares	7.50
Within-group sum of squares	6.91
F	1.41
p	**0.035**

***p* value**

	NT	TILL
DIG	**0.0273**	0.1453
NT		0.1654

Fig. 4 Principal Coordinate Analysis (PCoA) based on Bray–Curtis similarity distance of OTUs with relative abundance > 0.1% of total (from 16S rDNA targets) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities of the olive orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Significant differences were detected by permutational MANOVA (PERMANOVA)

(18 weeks from the start of the trial, T3), compositional differences among the three treatments at both phylum and class level were negligible (Fig. 5A).

The phylogenetic analysis of reverse-transcribed 16S rRNA targets confirmed that the active prokaryotic community in the citrus orchard soil was primarily affected by time-dependent changes; particularly in NT, no marked variations were observed (Fig. 5B). However, selective compositional shifts were noticed, especially in DIG treatments: as well as seen with 16S rDNA targets (that is, a temporary rise of γ-*Proteobacteria* and a short-lived reduction of Actinobacteria and Gemmatimonadetes), marked and transient compositional changes were observed shortly after anaerobic digestate addition. Indeed, there was an immediate increase in the relative abundance of Firmicutes; a decline in the relative abundance of β-*Proteobacteria*; an initial reduction of δ-*Proteobacteria* followed by a steady increase at the final stage (Fig. 5B). Conventional tillage did not exert a strong and immediate effect on the metabolically active soil prokaryotic community, which in most cases fluctuated across times. However, 7 weeks (T2) after the treatment, the relative abundance of α-*Proteobacteria* (which was still high at the final stage) and Firmicutes (mainly *Bacilli*) increased, whereas those of Actinobacteria and Planctomycetes decreased (Fig. 5B).

The ammonia-oxidizing archaeal class *Nitrososphaeria* (phylum Thaumarchaeota) was surveyed in the total but not in the active bacterial community, and showed a marked increase in relative abundance 7 weeks from the start of the trial (T2), both in TILL (+62%) and, at higher extent (+187%), in DIG treatments, before slowing down to initial values at T3 (Fig. 5A, B).

Also in the citrus orchard, soil time exerted a significant effect ($P < 0.05$) on all diversity indices calculated either for the total or the active soil prokaryotic communities (Table 5). Moreover, soil management and soil management × time interaction affected the variability of the Chao1 index of both total and active soil prokaryotic communities.

Focusing on the metabolically active community, it was found that the Shannon–Wiener diversity and species richness were significantly influenced by the management, whereas soil management × time interaction affected richness and evenness (Table 5).

Same as in the olive orchard soils, the output of Mantel test evidenced a stronger correlation between soil chemical properties and the total prokaryotic soil community as compared to the active prokaryotic soil community (Fig. 6). The total community significantly correlated with TSN, while the active community was significantly correlated with only EC and TSN. The first two CCA axes explained 81.12% and 83.15% of the total variance, respectively, for the total and the metabolically active prokaryotic community (Fig. 6). Both total and active communities formed distinct clusters grouped by sampling time, while weakly grouped by soil management. With regard to the active community, samples from the digestate-treated soil showed separate clusters at T1 and T2.

Results from the PCoA of the total and the metabolically active prokaryotic communities were visualized by constructing an ordination biplot PC1 *vs* PC2, which accounted for 41.60% (total community) and 46.35% (active community) of the total explained variance (Fig. 7). Same as in the olive orchard, both total and active communities clustered differently under different sampling times. PERMANOVA results confirmed a significant global effect of the sampling time (total community: $F = 6.79$, $P < 0.001$; active community $F = 7.16$, $P < 0.001$). In the citrus grove soil, management treatments did not exert any significant effect on both the total and the active prokaryotic soil communities.

Compared to soil of the olive orchard, network complexity was markedly different in the citrus orchard soil with a higher average degree, and a higher number of nodes and edges (Supplementary Fig. 7). Furthermore, the highest network complexity was found in NT and lowest network complexity was found in TILL, revealing that the network complexity gradually decreased from NT to DIG and to the TILL treatment (with an average degree of 115.52 in NT, 104.66 in DIG, and 57.55 in TILL). Proteobacteria followed by Actinobacteria, Planctomycetes, and Acidobacteria were the most abundant bacterial taxa under all management practices. There was not much difference in taxonomic composition of networks amongst different treatments.

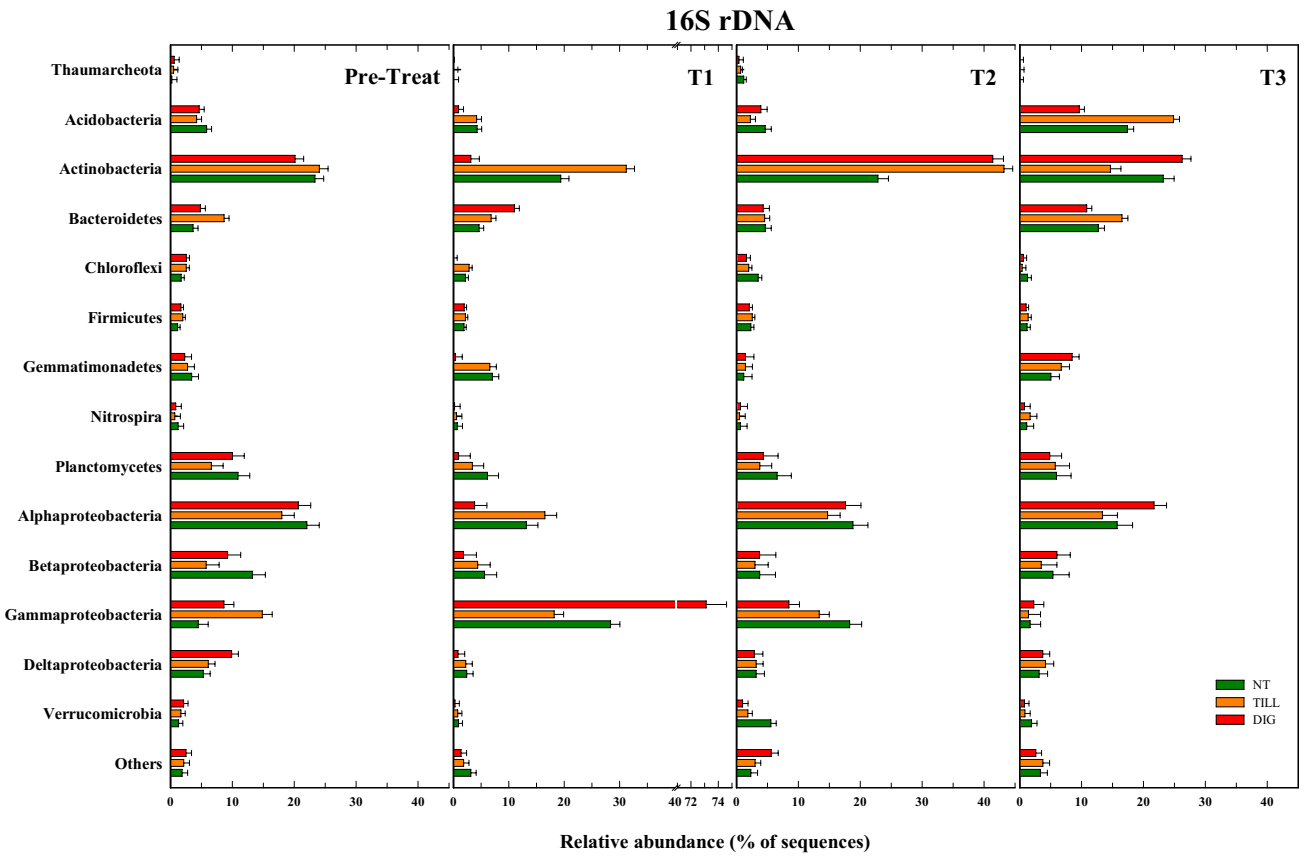
The main contributing OTUs to the dissimilarity of the total soil prokaryotic community (SIMPER test, Supplementary Table 2) were the chemolithoautotrophic ammonia-oxidizing archaeal microorganism "*Candidatus* Nitrosocosmicus" (Thaumarchaeota), which sharply increased after 7 weeks with the largest relative abundance found in DIG soil; an unidentified *Parcubacteria* group bacterium and the actinobacterial *Dactylosporangium solaniradicis*, detected only at the T1 and T2 sampling, that showed a transient increase in their relative abundance especially in tilled (TILL, DIG) plots; the filamentous bacterium EU25 (Chloroflexi) found more abundant at the T2 sampling, especially in TILL and DIG soils, and then declining considerably; *Nitrospira japonica* (Nitrospira), which fluctuated across treatments and over time being more abundant at the final stage; *Bacillus* spp. (Firmicutes), whose relative abundance permanently increased soon after the treatment, especially in TILL and DIG treatments; the acidobacterial *Acidobacteria bacterium WSR12*, which was always more abundant in no-tilled soil; and finally the α-proteobacterial *Skermanella* spp., whose relative abundance markedly increased over time mainly in tilled plots. Moreover, OTUs which contributed the most to discriminate the active prokaryotic community in this soil were the two γ-proteobacterial *Pseudomonas* spp. and *Pseudomonas formosensis*, which showed an immediate and short-lasting increase in the digestate-treated soil (same as observed in the olive cropped soil); the α-proteobacterial *Skermanella* spp. and two members of *Bacillus* spp. (Firmicutes) occurring in all treatments across samplings, but particularly in the TILL treatment at T2; as well as these, the two α-proteobacterial *Microvirga* spp. increased over time in all treatments and were found particularly represented in both tilled (TILL, DIG) soils; finally, actinobacterial *Acidimicrobidae bacterium* and *Nocardioides* spp. varied considerably across time with larger increments found in the NT treatment.

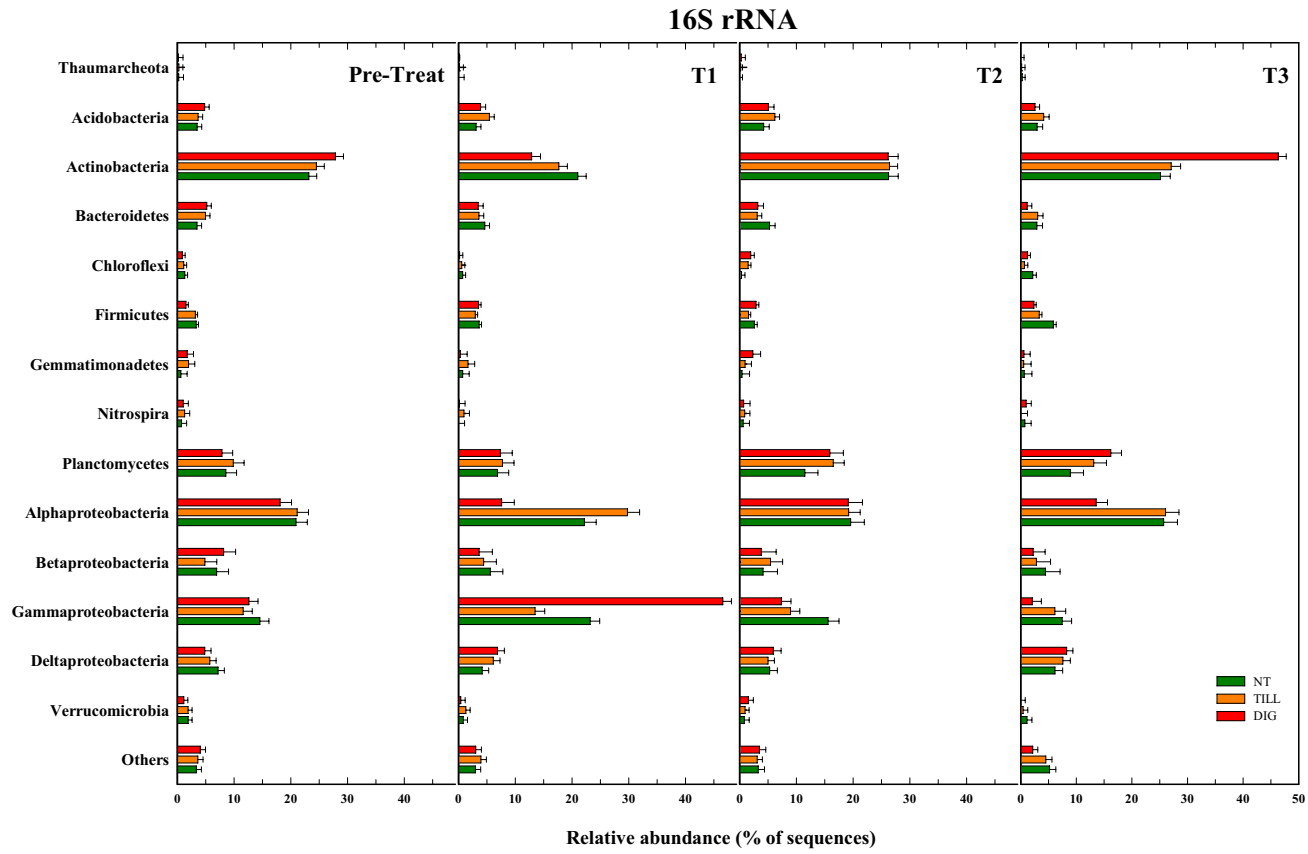
Discussion

Soil is a complex heterogeneous ecological system, generally poor in nutrients and energy sources, and harbors a wide spectrum of microorganisms residing in discrete microhabitats unevenly distributed through the soil fabric where they live and function (Young and Ritz 2005). This becomes particularly important in agricultural soils where variation in physical and chemical properties can exert a considerable influence on microbial activity, growth, and community composition, and hence on the functioning and the fertility of the soil. Acknowledging the fact that in the Mediterranean area conventionally managed orchard soils are particularly vulnerable to degradation, gaining a better understanding of the role and function of microorganisms in regulating soil responses to improved management practices becomes crucial to promote the sustainable land use of these highly susceptible agricultural areas.

Phylogenetic diversity of indigenous soil bacteria

In both orchard soils, we found that the resident total bacterial community was largely affiliated with ubiquitous Actinobacteria and Proteobacteria (especially copiotrophic


16S rDNA
Pre-Treat
T1
T2
T3
Thaumarcheota
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospira
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Verrucomicrobia
Others
NT
TILL
DIG
0 10 20 30 40
0 10 20 30 40 72 74
0 10 20 30 40
0 10 20 30 40
Relative abundance (% of sequences)


16S rRNA
Pre-Treat
T1
T2
T3
Thaumarcheota
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospira
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Verrucomicrobia
Others
NT
TILL
DIG
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40 50
Relative abundance (% of sequences)

◂**Fig. 5** Changes in relative abundances (mean ± SD, *n* = *3*) of total (**A**, from 16S rDNA templates) and metabolically active (**B**, from reverse-transcribed 16S rRNA targets) prokaryotic taxa in the citrus orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Only are shown those taxa with an average relative abundance > 1%

α- β-, and γ-*Proteobacteria*), which are largely represented in agricultural soils (Janssen 2006) including Mediterranean orchard soils (Curiel Yuste et al. 2014; Siles et al. 2014; Bastida et al. 2017; Rajhi et al. 2020). These taxa are widely distributed, inhabit nutrient-rich soils, participate in C, N, and S cycling, drive organic matter turnover and decomposition, regulate soil–plant interactions, release extracellular enzymes, and produce secondary metabolites (Spain et al. 2009; Fausto et al. 2018). Planctomycetes (which include also anaerobic ammonium-oxidizing bacteria) and Bacteroidetes (which have a copiotrophic lifestyle) represented the third and the fourth abundant taxa in both orchard soils, and were less represented than in Fausto et al. (2018) and Piazza et al. (2019), or more abundant than in Siles et al. (2014) in studies done in Mediterranean agricultural soils. We hypothesize that the relative abundance of members of Bacteroidetes is likely due to their ability to tolerate drought/heat waves occurring in semi-arid agroecosystems (Bastida et al. 2017), while high temperatures usually occurring during the summer period could have promoted the increase of Planctomycetes (up to 9%) observed in both tested soils (Wiegand et al. 2018). Even though here underrepresented (< 5%) than in other soils (~ 20%; Janssen 2006), low pH conditions were expectedly conducive to Acidobacteria in the olive orchard soil, but not in the slightly alkaline citrus one. Dry and acidic soil conditions in the olive soil may have favored *Blastocatellia* and *Solibacteres*. Even though Acidobacteria were found to be generally more abundant in C-poor bulk soils, not all members of this group are oligotrophic (Fierer et al. 2007). In addition, they are associated with Proteobacteria suggesting that the ratio between Proteobacteria and Acidobacteria may provide a better insight into the general nutrient status of soils (Smith et al. 2001), for instance following organic amendment or fertilization practices. Oligotrophic and widely distributed Gemmatimonadetes are generally poorly represented in soil (< 2.0%; Janssen 2006), but exceptions have been observed in Mediterranean arable soils (Siles et al. 2014), where an adaptation to low soil moisture (DeBruyn et al. 2011; Moroenyane et al. 2018) can bring about a population increase as observed here, especially in the clay soil. It is also worth noting that acidic soil conditions constrained *Longimicrobia*, which were surveyed only in the slightly alkaline (citrus) soil. Although being highly variable in soil (Janssen 2006), the relative abundance of the oligotrophic phylum Chloroflexi was in line with reported values (Castañeda and Barbosa 2017; Fausto et al. 2018). The good soil aeration of the sandy loam (citrus) soil favored aerobic *Chloroflexia* and *Ktedonobacteria*, which were restricted in the poorly aerated clay (olive) soil. Interestingly, the selective detection of members of the class *Verrucomicrobiae*, which accounted for the greater relative abundance of the phylum Verrucomicrobia, occurred in the citrus but not in the olive grove soil. Generally, they constitute an average of 7% of the total community in agricultural soils (Moroenyane et al. 2018), and they play an important role in biogeochemical cycling processes being responsive to soil management and nutritional regimes (Fausto et al. 2018; Piazza et al. 2019). Marked differences between the two orchard soils were observed in the class composition of phylum Firmicutes, despite the relative abundances were in line with what previously reported (Castañeda and Barbosa 2017; Fausto et al. 2018; Piazza et al. 2019). The ubiquitous in nature and common in soil members of class *Bacilli* (which can be either obligate aerobes or facultative anaerobes) were severely constrained by the physicochemical conditions (i.e., acidic pH, reduced aeration) occurring in the olive orchard soil. Finally, nitrite-oxidizing bacteria, such as Nitrospira, and ammonia-oxidizing archaea belonging to the phylum Thaumarchaeota (class *Nitrososphaeria*) were equally represented in both orchard soils.

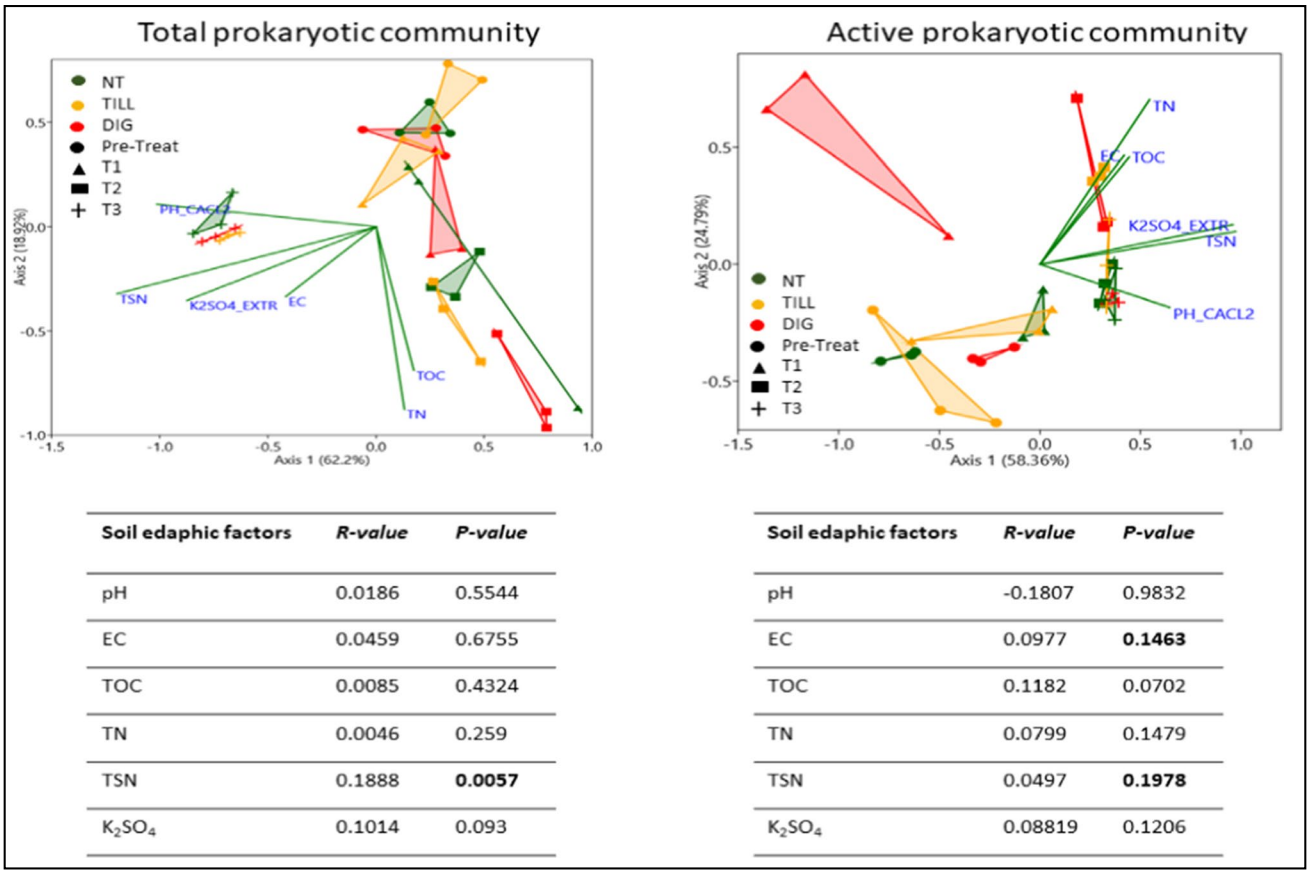
Even if a unifying procedure of cell lysis for DNA/RNA isolation from soil is advisable in comparative studies as to avoid potential limitations due to differing extraction efficiency (Nannipieri et al. 2020), finely optimized protocols are often needed to extract nucleic acids depending on soil properties using differing available commercial kits (van Elsas and Boersma 2011). In both soils, the total and active bacterial communities were not as distinctive as we would have expected, being the most relevant difference due to a reduction of active members of Chloroflexi and Gemmatimonadetes, and to a greater relative abundance of active members of the class *Bacilli* (Firmicutes). This latter finding confirms that members of this group are generally underrepresented in libraries of total bacterial communities, though they can be active components of soil bacterial communities, in line with what was first reported by Felske et al. (1998).

Phylogenetic diversity of anaerobic digestate bacteria

The anaerobic digestate was dominated by members of phyla Firmicutes (especially from classes *Clostridia* and *Bacilli*), and Bacteroidetes, thus corroborating what was previously reported (Hassa et al. 2018; Wei et al. 2020), whereas the relative abundance of Proteobacteria (with the methanothrophic γ-*Proteobacteria*) was low, depending also on mesophilic/thermophilic AD conditions. It is noteworthy that the active bacterial community markedly

Table 5 Changes in the α-diversity indices of total (from 16S rDNA targets) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities surveyed in the citrus orchard soil under different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 growing season. Different letters indicate significant differences within each sampling time (Tukey's HSD at $P<0.05$). Significant effects due to soil management (M), time (T), and their interaction are presented as F-values and level of significance (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) estimated by two-way ANOVA (management × time)

Molecular pool	Index	Sampling time											
		Pre-treat			T1			T2			T3		
		NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG	NT	TILL	DIG
16S rDNA	H′	2.3 ± 0.3	1.9 ± 0.1	1.9 ± 0.1	3.6 ± 0.4	3.8 ± 0.2	3.7 ± 0.1	3.4 ± 0.1	4.0 ± 0.2	3.4 ± 0.2	5.0 ± 0.1	5.0 ± 0.1	5.0 ± 0.1
		T: $F_{3,24}=129.1$***											
	S	11.0 ± 3.4	6.7 ± 0.3	6.7 ± 0.7	46.6 ± 20.7	44.7 ± 9.2	40.0 ± 1.0	31.0 ± 3.6	57.3 ± 12.0	32.0 ± 5.6	172.3 ± 21.4	165.3 ± 12.7	167.0 ± 11.6
		T: $F_{3,24}=122.30$***											
	E	1.00 ± 0.00	1.00 ± 0.00	0.99 ± 0.01	0.99 ± 0.01	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.99 ± 0.00	0.97 ± 0.00	0.98 ± 0.00	0.98 ± 0.00
		T: $F_{3,24}=22.47$***											
	Chao1	53 ± 17	26 ± 2	20 ± 1	474 ± 148	552 ± 118	368 ± 56	299 ± 10^{b}	659 ± 28^{a}	235 ± 14^{b}	646 ± 12^{b}	721 ± 14^{a}	757 ± 19^{a}
		M: $F_{2,24}=7.01$**; T: $F_{3,24}=68.01$***; M × T: $F_{6,24}=3.90$*											
16S rRNA	H′	3.0 ± 0.1	2.6 ± 0.1	3.1 ± 0.2	4.8 ± 0.3	4.5 ± 0.1	4.7 ± 0.3	5.0 ± 0.1	4.1 ± 0.3	3.8 ± 0.4	4.6 ± 0.1^{b}	4.1 ± 0.2^{b}	4.8 ± 0.1^{a}
		M: $F_{2,24}=6.12$**; T: $F_{3,24}=42.94$***											
	S	20.7 ± 0.3	13.3 ± 1.9	22.7 ± 5.7	156.3 ± 52.0	98.7 ± 7.4	188.7 ± 36.5	209.0 ± 9.3^{a}	100.3 ± 30.8^{b}	66.7 ± 21.5^{b}	106.3 ± 11.2^{ab}	74.3 ± 1.0^{b}	151.7 ± 11.2^{a}
		M: $F_{2,24}=5.67$**; T: $F_{3,24}=19.70$***; M × T: $F_{6,24}=4.35$**											
	E	0.99 ± 0.01	1.00 ± 0.00	1.00 ± 0.00	0.98 ± 0.01	0.98 ± 0.00	0.90 ± 0.04	0.94 ± 0.01	0.92 ± 0.03	0.94 ± 0.02	0.98 ± 0.00	0.98 ± 0.00	0.96 ± 0.02
		T: $F_{3,24}=8.81$***; M × T: $F_{6,24}=2.52$*											
	Chao1	217 ± 7^{a}	74 ± 4^{c}	168 ± 15^{b}	646 ± 44^{a}	463 ± 45^{b}	682 ± 49^{a}	687 ± 8^{a}	336 ± 89^{b}	303 ± 15^{a}	523 ± 6	497 ± 76	658 ± 46
		M: $F_{2,24}=15.34$**; T: $F_{3,24}=64.61$***; M × T: $F_{6,24}=7.31$**											

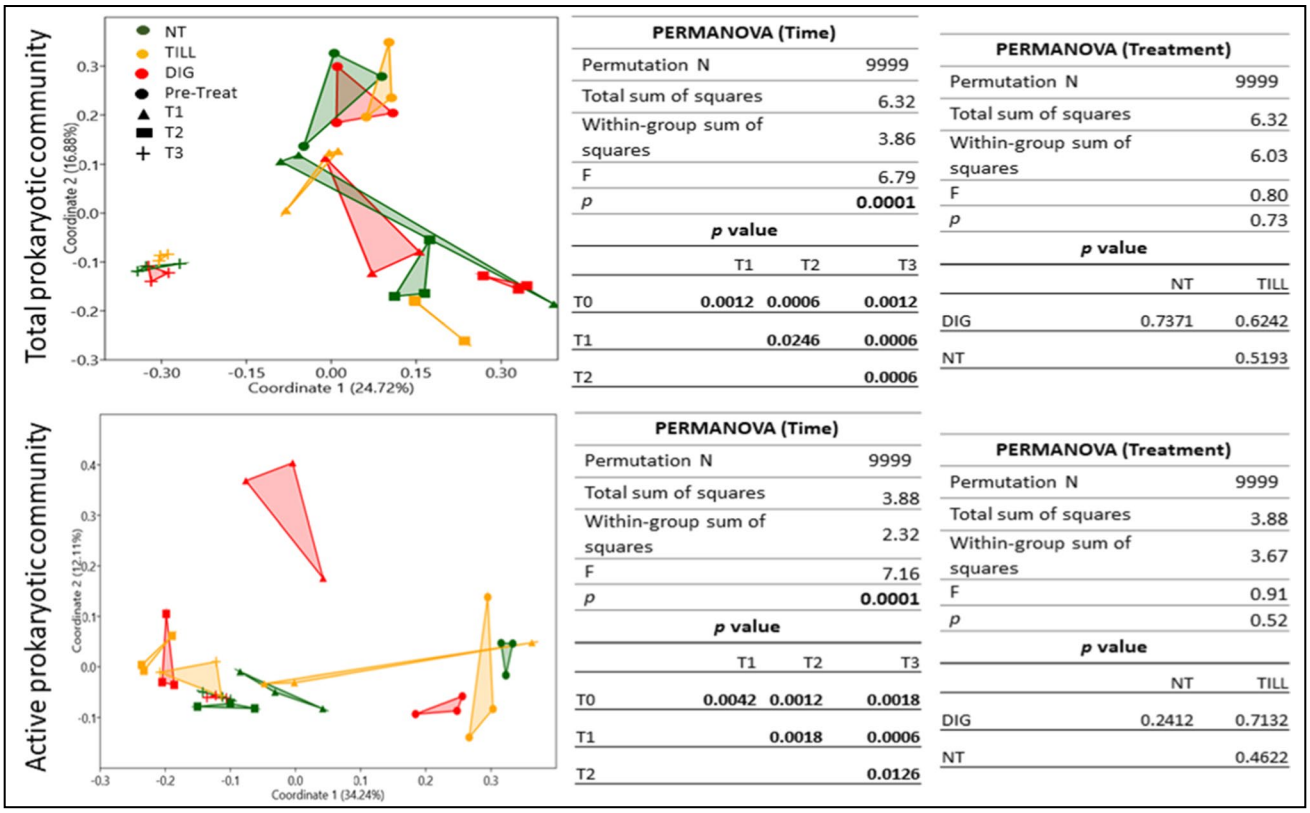


Soil edaphic factors	*R-value*	*P-value*
pH	0.0186	0.5544
EC	0.0459	0.6755
TOC	0.0085	0.4324
TN	0.0046	0.259
TSN	0.1888	**0.0057**
K_2SO_4	0.1014	0.093

Soil edaphic factors	*R-value*	*P-value*
pH	-0.1807	0.9832
EC	0.0977	**0.1463**
TOC	0.1182	0.0702
TN	0.0799	0.1479
TSN	0.0497	**0.1978**
K_2SO_4	0.08819	0.1206

Fig. 6 Canonical corresponding analysis based on Bray–Curtis similarity distance of OTUs with relative abundance > 0.1% of total (from 16S rDNA templates) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities of the citrus orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Significant differences were detected by Mantel test (Pearson correlation coefficients)

differed from the total bacterial community which included active, potentially active, dormant or inactive, and dead microbial states (Blagodatskaya and Kuzyakov 2013), and it was characterized by a greater phylogenetic diversity. Needless to say, community profiling based on 16S rRNA gene sequence targets bacterial groups from a very broad range of physiological states, whereas the phylogenetic diversity assessed by targeting 16S rRNA molecules represents the active bacterial community. We hypothesize that the consistent compositional difference observed in the active community was likely due to the competitive proliferation of copiotrophic fast-growing Proteobacteria and Actinobacteria (as well as other competitive colonizers such as members of taxa Planctomycetes or Chloroflexi) over the nutrient-rich organic matrix once exposed to environmental conditions (characterized by a different moisture, temperature, air composition, radiation, etc.) after the AD process. This finding suggests that anaerobic digestates are highly complex microbial systems constituted by a variety of chemically and physically heterogeneous micro-niches, rich in nutrients, and hosting a wide bacterial diversity, which dynamically change under changing environmental conditions during the post-treatment stage. Noteworthy, the number of active class members observed in the anaerobic digestate was affiliated with class *Clostridia*, which include obligate anaerobes and agent of soft rot disease in plants (Campos et al. 1982; Allen et al. 2009); class *Flavobacteriia*, which comprise commensal bacteria and also opportunistic pathogens for humans and other mammals (Hahnke et al. 2016); class *Erysipelotrichia* and *Chitinophagia*, which are often found in gut microbioma; class *Coriobacteriia*, which have been implicated with human diseases. To sum up, besides human pathogens such as *Salmonella* spp., *Escherichia coli*, *Listeria* spp., and *Clostridium perfringens* which are currently being monitored (Goberna et al. 2011; Nkoa 2014; Insam et al. 2015) and regulated by most national and European legislations (EU Regulation n. 1009/2019), it is advisable to extend our monitoring to a wider range of potentially harmful microbial groups or adopt proper post-treatment measures to reduce risks for



PERMANOVA (Time)

Permutation N	9999
Total sum of squares	6.32
Within-group sum of squares	3.86
F	6.79
p	**0.0001**

***p* value**

	T1	T2	T3
T0	**0.0012**	**0.0006**	**0.0012**
T1		**0.0246**	**0.0006**
T2			**0.0006**

PERMANOVA (Treatment)

Permutation N	9999
Total sum of squares	6.32
Within-group sum of squares	6.03
F	0.80
p	0.73

***p* value**

	NT	TILL
DIG	0.7371	0.6242
NT		0.5193

PERMANOVA (Time)

Permutation N	9999
Total sum of squares	3.88
Within-group sum of squares	2.32
F	7.16
p	**0.0001**

***p* value**

	T1	T2	T3
T0	**0.0042**	**0.0012**	**0.0018**
T1		**0.0018**	**0.0006**
T2			**0.0126**

PERMANOVA (Treatment)

Permutation N	9999
Total sum of squares	3.88
Within-group sum of squares	3.67
F	0.91
p	0.52

***p* value**

	NT	TILL
DIG	0.2412	0.7132
NT		0.4622

Fig. 7 Principal Coordinate Analysis (PCoA) based on Bray–Curtis similarity distance of OTUs with relative abundance > 0.1% of total (from 16S rDNA templates) and metabolically active (from reverse-transcribed 16S rRNA targets) prokaryotic communities of the citrus orchard soil across different treatments (NT, TILL, DIG as in M&M) at four sampling times (6 days before (Pre-treat) and then 2 days (T1), 7 weeks (T2), and 18 weeks (T3) after the treatment event) during the 2016/2017 cropping season. Significant differences were detected by permutational MANOVA (PERMANOVA)

the broad environment and human health, thus ensuring safe handling and management of anaerobic digestates.

Bacterial compositional responses to soil incorporation of solid anaerobic digestate

Even though soil incorporation of a solid anaerobic digestate stimulated marked shifts in the phylogenetic composition and relative abundance of major bacterial taxa, much perturbation was exhausted over the short term (less than 7 weeks). This finding reveals that the native soil bacterial community composition was remarkably resilient to the addition of the digested by-product (as also shown by the Venn diagram), and the altered picture of the molecular profile of soil microbiota reverted to previous conditions within the first weeks following the organic amendment. It also corroborates the short-lasting effects of soil addition of anaerobic digestate as previously reported (Nkoa 2014; Abubaker et al. 2015), and due to (1) the occurrence of most potential changes in bacterial diversity within the first weeks after the organic amendment (Siles et al. 2014), and (2) these changes are primarily due to the physicochemical properties of the organic matrix rather than to newly introduced microorganisms, which are easily and rapidly outcompeted by members of the autochthonous soil microbiota (Podmirseg et al. 2019). Don et al. (2017) named this process as the "home-field advantage." It should be also noted that the compositional responses were more evident at level of the active than the total bacterial community (thus confirming the hypothesis 1), and the magnitude (but not the persistence) of the changes depended on the soil type (partially supporting hypothesis 2). The addition of the easily degradable organic materials to soil (i.e., the remarkable increase observed in soluble C and N substrates following digestate incorporation, as well as the increase in C_{org} and N_t), strongly stimulated the growth of *r*-strategists. In particular, digestate incorporation stimulated the members of the group γ-*Proteobacteria* in both soils and *Bacilli* in citrus orchard soil, both known for their heterotrophic lifestyle and ability to grow on a wide range of organic substrates (Spain et al. 2009), including fresh substrates (Fierer et al. 2007). This hypothesis was corroborated by the Simper test that allowed to identify *Pseudomonas* spp. and *Pseudomonas formosensis* as discriminating γ-*proteobacterial* members of

the total (olive orchard soil) and the active (olive and citrus orchard soils) bacterial communities, and *Bacillus* spp. as discriminating Firmicutes members of the total and active bacterial communities in the citrus orchard soil. Interestingly, the late successional change towards OTUs affiliated with Actinobacteria found in the olive but not in the citrus orchard soils, and confirmed by identifying representative actinobacterial K-strategist species such as *Dactylosporangium solaniradicis* and *Arthrobacter* spp. in the total and active bacterial communities, respectively, suggests that a prolonged and slow-proceeding degradation process of more recalcitrant organic substrates occurred in the clay soil. This finding corroborates Badagliacca et al. (2020), who observed that the fine-textured (olive orchard) soil showed an increased aggregates stability, higher microbial biomass and C-use efficiency, and a longer-lasting release of soluble C and N forms than the citrus orchard soil, where the coarse-textured conditions fostered the rapid depletion of the newly added organic substrates. It can be also argued that digestate-induced short-term changes in chemical soil properties (except pH which remained practically unaffected in both orchard soils due to the buffering action of the high clay content, olive, or the calcium carbonate system, citrus) could have affected the bacterial community composition (Nkoa 2014; Insam et al. 2015). The expected increase in EC observed in both digestate-treated soils confirmed what previously reported by Gómez-Brandón et al. (2016), and it was not the main factor affecting the soil bacterial community composition.

Bacterial compositional responses to soil tillage

Strong seasonal shifts in soil bacterial communities were the most prominent finding observed in both orchard soils managed under conventional or conservative (no-tillage) practice, thus supporting what previously reported (Bevivino et al. 2014; Curiel Yuste et al. 2014; Frenk et al. 2015), and likely it depended on the fluctuating air temperature and rainfall regimes, which are typical of Mediterranean agroecosystems. The effect of a differing tillage practice on soil bacterial α-diversity was not clearly detected, as expected, and only the Chao1 index responded distinctly to no-tillage, being positively affected in most cases albeit for a limited period of time. However, the phylogenetic changes limited to a few bacterial taxa—similar at level of both total and active soil bacterial community (the hypothesis 1 is not confirmed)—were observed under conventional tillage, despite the contrasting properties and crops of the two orchard soils (Badagliacca et al. 2020). Yet, the sensitivity of Chloroflexi, and at a lesser degree of Verrucomicrobia, to tillage-induced disturbance was also confirmed (Wang et al. 2019). On the contrary, soil tillage induced a transient increase of the relative abundance of members of *Nitrososphaeria* (phylum Thaumarchaeota), a group of archaeal ammonia oxidizers whose growth is stimulated by the addition of organic acids, as also confirmed by the greater increase observed in digestate-treated plots. This finding was in agreement with Piazza et al. (2019), but in contrast with what reported by Dorr de Quadros et al. (2012).

A higher bacterial diversity was generally found under conservative than under conventionally tilled systems (Sengupta and Dick 2015; Montanaro et al. 2017). However, this may not be true in Mediterranean orchard soils where conflicting results have been observed (Castañeda and Barbosa 2017; Sofo et al. 2019; Vignozzi et al. 2019). Factors such as soil properties, tillage intensity, climatic conditions, geographic location, and observation time together with the high variability of soil microbial communities in time and space make the assessment of small changes in microbial composition difficult to be detected (Girvan et al. 2003; Boukhdoud et al. 2016; de Graaf et al. 2019).

The late successional change towards OTUs affiliated with slow-growing Acidobacteria was corroborated by the increase in the *Bacterium Ellin 6099* in the fine-textured olive orchard soil. On the other side, the rapid exhaustion of easily degradable organic substrates due to the tillage-induced soil disturbance stimulated the transient (up to 7 weeks) growth of copiotrophic γ-*proteobacterial* members such as *Pseudomonas* spp. and *Pseudomonas formosensis*. The tillage-induced changes in the relative abundance of *α-Proteobacteria* were confirmed by the fluctuations in *Skermanella* (in both soils) and *Microvirga* (only in citrus cropped soil), bacterial genera inhabiting Mediterranean soils (Siles et al. 2014). The relative abundance of *Bacillus* spp. was significantly high, especially in the conventionally tilled citrus cropped soil during the summer samplings, likely due to the survival of endospores resistant to high air temperature and dry climate (Berendsen et al. 2015, 2016).

The 16S rRNA-based survey revealed the presence of *Escherichia* spp. in the metabolically active bacterial community of the olive but not of the citrus orchard soil. The result suggests that specific strains of this human/animal host-associated pathogen can be integrated into indigenous microbial communities in secondary, non-host-associated habitats such as soil, manure, water, or plants (van Elsas et al. 2011), and possibly digestates (Nkoa 2014; Insam et al. 2015; Ongeng et al. 2015). However, a direct link between anaerobic digestate incorporation and soil occurrence of *Escherichia* spp. was not demonstrated in our study. It may be possible that the human pathogen had already established as a member of the active bacterial community in the olive (but not in the citrus) orchard soil, where more favorable environmental conditions (i.e., acidic pH, poor aeration, high moisture, clay content, limiting nutrient content, etc.) could have promoted its survival and persistence (Gagliardi and Karns 2002; Jang et al. 2017). It is interesting to note

that the physical disturbance due to tillage, either combined or not with anaerobic digestate addition, decreased the proliferation of the pathogen in soil.

Conclusions

Incorporation of solid anaerobic digestate in both soils brought about an immediate (2 days) but short-lived (up to 7 weeks) compositional shift in the phylogenetic composition of either the total or the metabolically active prokaryotic communities. This change involved the increase in the relative abundance of copiotrophic *γ-Proteobacteria* accompanied by a decline of oligotrophic Acidobacteria, Gemmatimonadetes, and Verrucomicrobia, whose balance was certainly altered by the addition of easily decomposable organic substrates. Selective responses related to the soil type were observed for members from Actinobacteria, Chloroflexi, Firmicutes, and Planctomycetes, being generally more favored (or less constrained) in the moderately coarse alkaline (citrus) than in the fine-textured acid (olive) soil. In any case, the indigenous soil microbiota demonstrated resilience after the addition of solid anaerobic digestate, an organic by-product dominated by members of phyla Firmicutes, Bacteroidetes, and Deinococcus-Thermus, and by the archaeal class *Methanomicrobia* (Euryarchaeota), which did not integrate within the autochthonous soil communities. Moreover, digestate addition caused long-lasting effects on soil C and N dynamics (still noticeable up to 1 year after the treatment) that were controlled by soil textural and chemical properties (Badagliacca et al. 2020). Overall, this finding supports the feasibility of soil application of solid anaerobic digestate as a proper and safe measure to manage the fertility status in arable lands, provided functional responses and C and N balance are duly considered in relation to the recipient soil properties. Moreover, in conventionally tilled plots, easier access to soil organic resources due to the mechanical disturbance stimulated the transient increase of taxa with a copiotrophic lifestyle (i.e., Firmicutes, Bacteroidetes), which could have accounted for major C resource depletion observed in the moderately coarse (citrus) soil. Likewise, the increased aeration due to the tillage event could have favored the ammonia-oxidizer archaeal *Nitrososphaeria* (Thaumarchaeota). Conversely, no-tillage induced an opposite phylogenetic shift in the r/K-strategist balance, favoring the establishment of oligotrophic bacterial taxa such as Chloroflexi and Verrucomicrobia, which was consistent across the two sites and stable over the entire observation period, and could have contributed to preventing the overexploitation of soil resources as observed in conventionally tilled soils.

In this study, most prokaryotic diversity was captured by the Chao1 index rather than by the Shannon–Weaver index, richness and evenness, and the active community did differ consistently from the total prokaryotic community in tilled (either amended or not) but not in no-tilled soils. This latter finding corroborates what stated by Hobbie and Hobbie (2013) that in global soils, which are generally characterized by poor, oligotrophic conditions, the majority of microorganisms have a starving-survival lifestyle of dormancy which makes them rapidly responsive to external disturbance and help them survive in a competitive and changing environment.

Taken together, our results allowed us to capture management-induced changes in total and active communities besides naturally occurring seasonal fluctuations, and to relate them to transformation processes occurring in soil. It also true that the potential contribution to keystone processes due to rare taxa should be not overlooked and properly addressed in the metagenomic analysis of soil microbiota (i.e., by increasing sequencing depth) to improve our understanding of interactions between bacterial community composition and external disturbance (van Elsas and Boersma 2011).

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